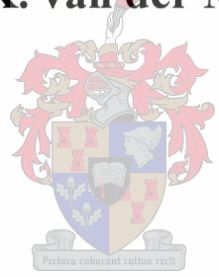
NCR-Sensitive Gene Expression and Regulation of Nitrogen Interconversion by *VID30* in *Saccharomyces cerevisiae*

George K. van der Merwe



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Supervisor: Professor H.J.J. van Vuuren

DECLARATION

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and has not previously, in its entirety or in part, been submitted at any university for a degree.

G.K. van der Merwe

SUMMARY

Saccharomyces cerevisiae uses the nitrogenous compounds in its environment selectively. The basis of this phenomenon is the transcriptional regulation of genes whose products are required for nitrogen catabolism. A rich nitrogen source represses the expression of genes required for the degradation of poor nitrogen sources via the action of the target of rapamycin (TOR) signaling cascade. If only a poor nitrogen source is available, these genes are derepressed. This process is known as nitrogen catabolite repression (NCR) or nitrogen regulation.

The *DAL1* and *DAL4* genes of *S. cerevisiae* are transcribed divergently from the 829 bp intergenic region. The five known UAS_{NTR} elements (*GATA1-5*) were mutated in the full context of the intergenic promoter. All five elements are required for the transcriptional activation of *DAL4*. The two elements most proximal to *DAL4* (*GATA4* and *GATA5*) contributed the most and the one most distal (*GATA1*) contributed the least to its expression. In contrast, three of the five elements (*GATA2-4*) are required for *DAL1* activation. In addition, analyses revealed that no single element is shared equally between these two genes. Predictions as to the function of known nitrogen-regulating elements based on their sequence and location proved to be inaccurate in some cases.

Mutation analyses of the three UIS_{ALL} elements present in the intergenic promoter region revealed that *UIS8*, which does not share a high degree of homology with the consensus UIS_{ALL} sequence, is required the most for transcriptional induction of both *DAL1* and *DAL4*. Also, *UIS7*, which shares the most similarity with the UIS_{ALL} consensus sequence, has the phenotype of a repressor-like element when mutated. These observations therefore portray the opposite phenotypes of what was expected.

We identified a regulator, Vid30p, which is required for the transcriptional response of *S. cerevisiae* in low ammonia conditions. Genetic analyses of the *vid30*Δ mutant indicate that Vid30p functions by regulating the expression of genes required for the production and degradation of glutamate. The transcription of *VID30* is NCR-sensitive, highly induced by low concentrations of ammonia, and rapamycin-sensitive. In addition, the *vid30*Δ mutant is hypersensitive to rapamycin, indicating that this protein is, directly or indirectly, controlled by the TOR signaling pathway.

OPSOMMING

Saccharomyces cerevisiae het die vermoeë om stikstofbronne vanuit die omgewing selektief te benut. Die basis van hierdie verskynsel is die transkripsionele regulering van gene wat vir proteïene kodeer wat stikstof katabolisme bemiddel. 'n Goeie stikstofbron onderdruk die transkripsie van gene wat met die degradering van swak stikstofbronne gemoeid is. Hierdie onderdrukking word deur die teiken-van-rapamisien (TVR)-seintransduksiepad bewerkstellig. Wanneer slegs 'n swak stikstofbron beskikbaar is, word hierdie gene geaktiveer. Hierdie verskynsel staan as stikstofkatabolietonderdrukking (SKR) of stikstofregulering bekend.

Die *DAL1*- en *DAL4*-gene van *S. cerevisiae* word divergent vanaf 'n 829 bp intergeniese area getranskribeer. Vyf UAS_{NTR}-elemente (*GATA1-5*) is in die volle konteks van die intergeniese promotor gemuteer. Al vyf elemente word vir *DAL4* transkripsionele aktivering benodig. Die twee elemente mees proksimaal tot *DAL4* (*GATA4* en *GATA5*) lewer die grootste bydrae tot *DAL4*-geenuitdrukking, terwyl die mees distale element (*GATA1*) die kleinste bydrae lewer. In teenstelling hiermee lewer slegs drie van die vyf elemente (*GATA2-4*) 'n noemenswaardige bydrae tot *DAL1* se uitdrukking. Nie een van die vyf elemente lewer 'n gelykwaardige bydrae tot die uitdrukking van *DAL1* en *DAL4* nie. Voorspellings betreffende die bydrae van die onderskeie UAS_{NTR}-elemente tot die uitdrukking van die *DAL1*- en *DAL4*-gene, gebaseer op die sekwens en die posisie van die element in die promotor, was meestal onakkuraat.

Die drie UIS_{ALL}-elemente in die intergeniese area is gemuteer en toon dat *UIS8*, wat nie 'n groot mate van homologie met die UIS_{ALL} konsensus sekwens deel nie, die mees kritiese element vir transkripsionele induksie van beide *DAL1* en *DAL4* is. *UIS7*, wat 'n hoër mate van homologie met die UIS_{ALL} konsensus sekwens deel, toon die fenotipe van 'n onderdrukkingselement wanner dit gemuteer word. Hierdie waarnemings is dus die teenoorgestelde van wat verwag is.

Ons het 'n reguleerder, Vid30p, geïdentifiseer wat benodig word vir die transkripsionele response van stikstofgereguleerde gene in lae konsentrasie ammonium. Genetiese analises van die *vid30*Δ mutant toon dat Vid30p funksioneer deur die transkripsie van gene gemoeid met die vorming en degradering van glutamaat te

reguleer. Die transkripsie van *VID30* is SKO-sensitief, word sterk deur lae konsentrasies ammonium geïnduseer, en is rapamisien-sensitief. Die *vid30*Δ mutant is ook hipersensitief vir rapamisien, wat aandui dat Vid30p, direk of indirek, deur die TVR-seintransduksiepad gereguleer word.

BIOGRAPHICAL SKETCH

George Karel van der Merwe was born on February 9, 1968 in Parow North, South Africa. He matriculated from Tygerberg High School, Parow North in 1985. He enrolled at the University of Stellenbosch in 1986 and received his B.Sc degree (Zoology and Human Physiology) in 1988. He added two additional majors (Biochemistry and Microbiology) by 1990. In 1991 he obtained his Hons.B.Sc. (Microbiology) *cum laude* from the University of Stellenbosch. He enrolled for a M.Sc. degree (Microbiology) at the University of Stellenbosch in 1992, which was upgraded to a Ph.D. (Microbiology) in 1995.

Mr. van der Merwe spent 10 months during 1995/96 in the laboratory of Prof. Terry Cooper at the Department of Immunology and Microbiology, University of Tennessee in Memphis, USA. He spent 15 months during 1998/99 at the Cool Climate Oenology and Viticulture Institute at Brock University in St. Catharines, Canada, under the guidance of Prof. Hennie van Vuuren. He has subsequently been working at the Wine Research Center at the University of British Columbia in Vancouver, Canada, also under the guidance of Prof. Hennie van Vuuren.

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CONTENTS

* *Each research paper was written according to the style of the journal in which it was published.*

INTRODUCTION

All organisms need nitrogen for growth and survival. *Saccharomyces cerevisiae* selectively utilizes a variety of nitrogenous compounds as sources of organic nitrogen. Rich nitrogen sources, such as ammonia and glutamine, are preferred above poor ones, such as proline and threonine (Cooper, 1982; Magasanik, 1992). The yeast senses environmental nitrogen and transduces the signal via the partially defined target of rapamycin (TOR) signaling cascade to the nucleus (Beck and Hall, 1999; Cardenas *et al.*, 1999; Hardwick *et al.*, 1999; Shamji *et al.*, 2000). A rich nitrogen supply represses the transcription of genes encoding proteins required for the catabolism of poor nitrogen sources, while a poor nitrogen supply results in the derepression of these genes.

The transcription of genes encoding proteins that function in the allantoin degradative system are nitrogen-sensitive and have been used as a model in studying the transcriptional response of nitrogen catabolic genes to different nitrogen environments. Promoter analyses of these and other nitrogen catabolic genes have identified three elements that function in a nitrogen-related manner. The UAS_{NTR} is required for transcriptional activation, the URS_{GATA} for repression, and the UIS_{ALL} for response to an inducer (Cooper, 1994). In addition, several transcription factors that influence the derepression of nitrogen-sensitive genes have been identified. These include the well-known GATA factors, Gln3p, Gat1p/Nil1p, Dal80p/Uga43p and Deh1p/Gzf3p/Nil2p (Coffman *et al.*, 1997; Cooper, 1994; Soussi-Boudekou *et al.*, 1997). It has since been shown that the TOR signaling cascade regulates the function of these proteins in response to the nitrogen supply (Beck and Hall, 1999; Cardenas *et al.*, 1999; Hardwick *et al.*, 1999; Shamji *et al.*, 2000). Proteins required for the induction of some nitrogen catabolic genes have also been identified. These include the Dal81p and Dal82p (Cooper, 1994).

The *DAL1* and *DAL4* genes encode the allantoinase and allantoin permease, respectively (Cooper, 1982). These proteins are required to initiate allantoin degradation. Interestingly, these genes are oriented in a head-to-head manner on the genome of *S. cerevisiae* and are possibly transcribed in opposite directions from an 829 base pair intergenic region. Five UAS_{NTR}- and two UIS_{ALL}-like elements have been

identified based on sequence homology with the consensus sequences reported for these elements (Buckholz and Cooper, 1991; Cooper, 1994; Yoo *et al.*, 1992).

The specific aims of this study included:

1. Analyzing the promoter-mediated transcriptional regulation of the *DAL1* and *DAL4* genes of *S. cerevisiae* by:
    a. Demarcating the promoter region(s) that support transcription of the *DAL1* and *DAL4* genes;
    b. Identifying and analyzing the contribution of known nitrogen-related *cis*-acting elements to the expression of each of these genes, and
    c. Determining the effect that known nitrogen-related transcriptional regulators have on the expression of these genes.
2. Identifying and genetically analyzing a novel gene whose protein product is required for nitrogen-related gene expression in *S. cerevisiae*

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LITERATURE REVIEW

CHAPTER 1

RNA Polymerase II transcription in eukaryotes

CONTENTS

INTRODUCTION

Three DNA-dependent RNA polymerases, RNA polymerase I, II, and III, transcribe the DNA of eukaryotic genomes to express its genetic information (Archambault and Friesen, 1993; Mosrin and Thuriaux, 1990). The process of transcription is complex, consisting of a series of events facilitated by multi-subunit protein complexes. All protein-coding genes in eukaryotic organisms are transcribed by RNA polymerase II (Pol II). The messenger RNA synthesized by this enzyme represents more than 90% of the *Saccharomyces cerevisiae* genome and, depending on the environmental conditions, comprises 1-5% of the total RNA produced (Goffeau *et al.*, 1996; Young, 1991).

Eukaryotic genomes are condensed in structures known as chromatin to enable packaging into the relatively small nucleus. This condensing influences all DNA-dependent processes, including transcription, as access to the DNA is restricted (Gregory, 2001). To add to the complexity of transcription, eukaryotes also control Pol II transcription in an effort to adapt to and survive in the environment. Some essential genes are constitutively expressed while others are regulated in a coordinate fashion in response to environmental conditions. Multiple layers of transcriptional regulation therefore exist to allow the organism accurate control of its gene expression (Kornberg, 1999; Lee and Young, 2000)

RNA POLYMERASE II TRANSCRIPTION

Pol II transcription starts with the recruitment of Pol II to the 5' untranslated region or promoter of a specific gene. Following promoter recognition, a pre-initiation complex (PIC) is formed, which melts the promoter DNA to form an open complex containing a 12-15 basepair (bp) single stranded DNA bubble. Transcriptional initiation and promoter clearance commences as the first phosphodiester bonds of the RNA molecule are formed. Pol II initially undergoes abortive elongation by repeatedly re-initiating transcription and releasing short RNA molecules. Transition from abortive elongation to productive elongation requires promoter clearance by the transcription complex and the transition of the transcription apparatus from an initiation to an elongation complex (Gralla, 1996; Hampsey, 1998; Lee and Young, 2000; Uptain *et al.*,

1997). All these processes are targets for transcriptional regulation. This review will focus on the promoter-related events.

Pol II transcription is controlled by two classes of transcription factors: the core transcription machinery, which includes Pol II and general transcription factors (GTFs), and the transcriptional regulatory proteins that permit or deny Pol II function (Muller, 2001). These proteins interact with each other and the promoters of genes to ultimately enable the regulated expression of a specific gene.

A Typical Pol II Promoter

Yeast promoters typically contain a core promoter and a regulatory region (Struhl, 1995). Core promoters contain the core elements, including TATA and Initiator elements, which are needed for the assembly of the PIC. The regulatory region, located upstream of the core elements, contains regulatory elements that control transcription. Regulatory elements include activating and repressing sequences, which are the targets for binding of transcriptional stimulatory or repressing proteins, respectively.

Core elements

The yeast TATA element has a consensus sequence of 5'-TATAAA-3' and is located 40 to 120 nucleotides upstream of the transcription initiation site (Struhl, 1995). This sequence is the binding site of the TATA binding protein (TBP). The TBP-TATA complex is pivotal for transcriptional initiation as it serves as the basis for the assembly of a PIC (Klein and Struhl, 1994). Single or multiple copies of this element are found in the majority of yeast Pol II promoters. However, some promoters do not contain this element and are known as TATA-less promoters. These promoters remain TBP-dependent (Hampsey, 1998).

Initiator elements (Inr), identified as the consensus sequence of 5'-PyPyA$_{+1}$NT/APyPy-3', are defined as DNA sequences that encompass the transcription initiation site and direct transcription initiation, either singly or in combination with TATAA (Smale, 1997). General transcription factors, regulatory proteins and Pol II itself, interact with Inr (Carcamo *et al.*, 1991; Li *et al.*, 1994; Hampsey, 1998).

Regulatory elements

Activating elements. Upstream activating sequences (UASs), upstream induction sequences (UISs), enhancers and locus control regions (LCRs) are DNA elements bound specifically by specific transcriptional activators. UASs normally function in close proximity of the core promoter and bind single activators. Once bound, activators facilitate the assembly of the PIC and increase transcription either by direct or indirect interaction with the GTFs (Hamsey, 1998; Lee and Young, 2000; Struhl, 1995).

The transcription of the inducer-responsive *DAL* and *DUR* genes in *S. cerevisiae* is induced in addition to being transcriptionally activated (Cooper *et al.*, 1987; Genbauffe and Cooper, 1986; Yoo *et al.*, 1985). The UIS element required for this response, UIS_{ALL}, has been identified and functions in addition to the UAS required for the activation of the *DAL* genes (Olive *et al.*, 1991; Rai *et al.*, 1999; van Vuuren *et al.*, 1991; Yoo *et al.*, 1989).

Enhancers and LCRs are DNA elements that contain multiple binding sites for diverse transcriptional activators. Both elements have a positive effect on gene expression by facilitating the interaction between specific activators, co-activators and the transcriptional machinery of target promoters (Blackwood and Kadonaga, 1998; Li *et al.*, 1999). In addition, LCRs have the ability to unfold locus-specific chromatin domains (Fraser and Grosveld, 1998). Enhancers function in a distance- and orientation-independent manner, while the function of LCRs is limited by these parameters. LCRs activate transcription independent of chromatin structure, while the function of enhancers is highly dependent on transcriptionally permissive chromatin (Blackwood and Kadonaga, 1998; Li *et al.*, 1999).

Homopolymeric dA-dT [poly (dA-dT)] elements are common features in yeast promoters that stimulate transcription as their intrinsic structure impairs nucleosome assembly and stability. These elements are not packaged in chromatin (Iyer and Struhl, 1995).

Repressing elements. Upstream repressing sequences (URSs or operators) and silencers are DNA elements that enable transcriptional repression. URSs specifically bind repressor proteins that negatively affect Pol II transcription. Like UASs, these elements are normally in close proximity to the core promoter. URSs generally have the greatest effect on transcription when located between the UAS(s) and the core

promoter (Johnson, 1995). Proteins that bind these elements normally inhibit transcription by having a negative effect on promoter-bound proteins. In contrast, silencers are DNA elements required for the nucleation of silent chromatin. These elements bind regulators that facilitate the formation of transcriptionally inert DNA in a promoter-independent manner (Kamakaka, 1997; Laurenson and Rine, 1992).

Divergent promoters

Divergently transcribed genes are known forms of gene organization in the *S. cerevisiae* genome. A relatively small intergenic region, usually smaller than 1000 bp, can separate two related genes oriented in a head-to-head manner. The intergenic region can serve as a common promoter that supports the transcription of these genes in opposite directions. Divergently transcribed gene pairs include *GAL1-GAL10*, *MAL6T-MAL6S*, *SPS18-SPS19* and *DIT1-DIT2* (Bell *et al.*, 1995; Coe *et al.*, 1994; Friesen *et al.*, 1997; Johnston and Davis, 1984). The *DAL1* and *DAL4* genes of *S. cerevisiae* encode the allantoinase and allantoin permease, respectively (Cooper, 1982). These genes are transcribed in opposite directions from an 829 bp intergenic region and therefore have the characteristics of a divergently transcribed gene pair.

Divergent promoters contain DNA elements that are shared by both genes. The UAS_G (Upstream Activating Sequence for Galactose) located in the *GAL1-10* intergenic region is required for the activated transcription of these genes in response to galactose (West *et al.*, 1984, Yocum *et al.*, 1984). Also, a single UAS_M (Upstream Activating Sequence for Maltose) in the *MAL6T-MAL6S* promoter mediates the induction of both genes in response to the presence of maltose (Yao *et al.*, 1994). In addition, two functional Mig1p binding sites are also present on either side of the UAS_M element. Mig1p is a negative regulator that mediates glucose repression (Hu *et al.*, 1995) and renders the *MAL6T-MAL6S* promoter sensitive to glucose repression.

Despite sharing the same intergenic region, divergently transcribed genes are not necessarily transcribed at similar levels. *GAL1* is expressed approximately four-fold higher than *GAL10* in both glucose and galactose (West *et al.*, 1984, Yocum *et al.*, 1984). The expression levels of *MAL6T* and *MAL6S* are comparable and extremely high under induced conditions (growth in maltose), while glucose severely represses the expression of both genes to low levels. However, under non-induced and non-repressed

conditions (growth in glycerol/ethanol) the expression of *MAL6T* is significantly lower than *MAL6S* (Levine *et al.*, 1992).

The Core Transcription Machinery

RNA Polymerase II

The core eukaryotic Pol II is a multisubunit complex of 12 proteins that are encoded by the essential *RPB1-12* genes (Lee and Young, 2000). This enzyme is responsible for DNA-dependent mRNA synthesis, but is unable to recognize promoters or initiate transcription independent of additional transcription factors. The active site for RNA synthesis is a cleft formed between the largest two subunits, Rpb1p and Rpb2p. The catalytic magnesium ion required for RNA synthesis also locates to this region (Cramer *et al.*, 2000). The other subunits contribute to mRNA synthesis by aiding transcriptional start site selection (Hull *et al.*, 1995), interactions with transcriptional regulators (Miyao and Woychik, 1998) and determining transcriptional elongation efficiencies (Powell and Reines, 1996).

Rpb1p contains a carboxy-terminal repeat domain (CTD) that consists of tandem repeats of a conserved heptapeptide sequences (Tyr-Ser-Pro-Thr-Ser-Pro-Ser), is highly conserved from yeast to humans and is essential for cell viability (Allison *et al.*, 1988; Corden, 1990; Young, 1991). The function of this domain is closely related to its phosphorylated state. Dephosphorylated CTD is associated with the PIC and is thought to interact with the TATA-binding protein (TBP) (Lu *et al.*, 1991; Usheva *et al.*, 1992), while phosphorylated CTD is found in the elongating transcription complex (Cadena and Dahmus, 1987; O'Brien *et al.*, 1994). Phosphorylation of the CTD disrupts the CTD-TBP interaction and results in the transition from open complex formation to promoter clearance (Usheva *et al.*, 1992; Valay *et al.*, 1995). This transition from transcription initiation to elongation also changes the co-factors associated with CTD. The SRB/Mediator complex, a component of the PIC, is tightly associated with the dephosphorylated CTD, while the elongator complex and RNA processing factors, components required for post-initiation events, are associated with the phosphorylated CTD (Kim *et al.*, 1994; Koleske and Young, 1995; McCracken *et al.*, 1997).

Two CTD kinases have been identified. Kin28p is a subunit of the TFIIH general transcription factor and phosphorylates CTD to allow promoter clearance and

transcriptional elongation (Dvir *et al.*, 1997). The deletion of *KIN28* results in a complete loss of Pol II transcription in yeast (Holstege *et al.*, 1998). The second CTD kinase, Srb10p, is a component of the SRB/Mediator complex that tightly associates with CTD (Liao *et al.*, 1995) and will be discussed later. Fcp1p, a CTD phosphatase that associates with TFIIF (Archambault *et al.*, 1997; Kobor *et al.*, 1999), dephosphorylates the CTD during transcriptional elongation. Pol II is thereby recycled to enter the next PIC (Cho *et al.*, 1999).

General transcription factors

Transcription initiation requires the recruitment of Pol II to the core promoter to form a PIC. This process requires a variety of GTFs that act at the core promoter. These include TFIIA, TFIIB, TFIID, TFIIE, TFIIF and TFIIH (Dvir *et al.*, 2001; Lee and Young, 2000; Orphanides *et al.*, 1996).

The assembly of a PIC starts with the binding of the TFIID complex to the TATA element. The TBP is a subunit of TFIID and binds the TATA element. A large TBP-associated factor (TAF), $TAF_{II}145/TAF_{II}130$, directly binds TBP (Moqtaderi *et al.*, 1996) and acts as the scaffold to which 11 distinct TAFs can bind to form the TFIID complex (Poon *et al.*, 1995; Reese *et al.*, 1994). $TAF_{II}145/TAF_{II}130$ also assists in transcriptional start site selection (Shen and Green, 1997). TAFs have an important role in transcription initiation when the Inr core element is used, either alone or in combination with TATA, for PIC formation (Martinez *et al.*, 1994; 1995). These proteins also have an important function in the transcriptional regulation of Pol II (Burley and Roeder, 1996; Verrijzer and Tjian, 1996).

TFIIA functions in close relation to TFIID. It enhances the binding of TFIID to the core promoter (Orphanides *et al.*, 1996), displaces repressor proteins from the TFIID complex (Ozer *et al.*, 1998b) and promotes transcriptional activation by interacting with various activators (Ozer *et al.*, 1998a; Ozer *et al.*, 1994; Yokomori *et al.*, 1994).

TFIIB interacts directly with TBP and Pol II, thereby recruiting Pol II to the core promoter (Buratowski *et al.*, 1989; Nikolov *et al.*, 1995). It bridges the gap between the TATA element and the transcriptional start site and is essential for start site selection (Pinto *et al.*, 1992). It also directly interacts with various activators, suggesting its recruitment to promoters by these proteins (Lin *et al.*, 1991; Roberts *et al.*, 1993).

TFIIF consists of three subunits that tightly associate with Pol II (Henry *et al.*, 1994). It stabilizes the PIC (Conaway and Conaway, 1993; Greenblatt, 1991), functionally interacts with TFIIB to accurately select the transcriptional start site (Sun and Hampsey, 1995), and enhances the efficiency of elongation (Bengal *et al.*, 1991; Izban and Luse, 1992).

The function of TFIIE is closely related to that of TFIIH (Li *et al.*, 1994). TFIIE contributes to the unwinding and melting of the promoter DNA and is therefore essential for the transcriptional initiation and promoter clearance of the Pol II complex (Kuldell and Buratowski, 1997).

The TFIIH multisubunit complex contains at least three enzymatic activities: DNA-dependent ATPase, ATP-dependent helicase and a CTD kinase (Feaver *et al.*, 1991; Roy *et al.*, 1994; Schaeffer *et al.*, 1993). Open PIC formation requires the ATP-dependent helicase activity, ATP and TFIIE (Guzman and Lis, 1999; Holstege *et al.*, 1996; Wang *et al.*, 1992). The CTD kinase of TFIIH, Kin28p, phosphorylates the CTD to enable promoter clearance (Usheva *et al.*, 1992; Valay *et al.*, 1995). In addition, TFIIH promotes the formation of productive elongation complexes after promoter clearance (Dvir *et al.*, 1997). Thus, TFIIH is essential for the conversion of a PIC into a productive elongating complex.

Yeast Pol II holoenzyme

Several models for the assembly of a PIC have been proposed. These range from the stepwise assembly of the individual GTFs and Pol II to form a PIC (Buratowski *et al.*, 1989; Roeder, 1991) to the existence of a very large pre-assembled Pol II complex known as the Pol II holoenzyme (Kim *et al.*, 1994; Koleske and Young, 1994). Reconstituted transcription assays showed that purified holoenzymes respond to transcriptional activators, while highly purified core Pol II and GTFs do not (Koleske and Young, 1994; Sayre *et al.*, 1992). The holoenzyme generally contains core Pol II, most of the remaining GTFs and the SRB/Mediator complex (Fig. 1). These proteins associate prior to recruitment to the core promoter (Kim *et al.*, 1994; Koleske and Young, 1994). Collectively these observations favor the model of the holoenzyme being recruited to the core promoter during PIC assembly.

Several forms of the holoenzyme have been reported. Different complexes generally vary in the GTFs and/or regulatory complexes that associate with it. Some holoenzyme complexes lack only TBP and TFIIE (Koleske and Young, 1994), others lack TBP, TFIIB and TFIIE (Koleske *et al.*, 1996), while a final group lack all the GTFs except TFIIF (Kim *et al.*, 1994). Also, holoenzyme complexes have been isolated that either contains or lack the SWI-SNF chromatin remodeling complex (Kim *et al.*, 1994; Wilson *et al.*, 1996).

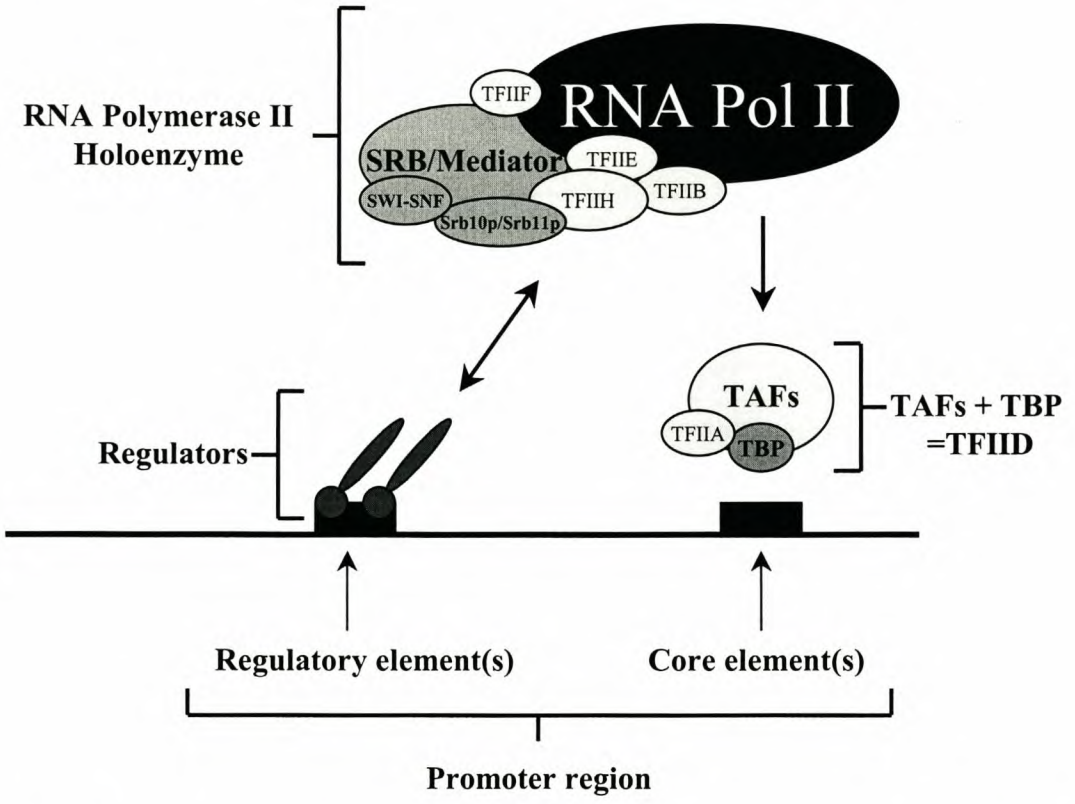

Fig. 1. Model for the yeast Pol II holoenzyme recruitment to the core promoter (adapted from Holstege *et al.*, 1998).

Regulatory Proteins

Two classes of regulatory proteins are involved in transcription regulation. Transcriptional activators stimulate transcription by enhancing the function of the transcription apparatus. Repressor proteins function at different levels to oppose activators and therefore repress transcription.

Activators

Yeast contains a variety of transcriptional activators that stimulate the expression of its target genes after binding to specific UASs in target promoters (Fig. 2). Activators are modular proteins typically consisting of two functionally distinct, and usually physically separable, domains. The DNA-binding domain is required to sequester/anchor the activator to its cognate UAS. This domain contains one of several known eukaryotic DNA-binding motifs, which include homeodomains, zinc fingers, bZIP and bHLH domains (Struhl, 1989; 1995). Activation domains facilitate the recruitment or stimulate the activity of the core transcription apparatus, thereby increasing the expression of their target genes (Ptashne and Gann, 1997; Triezenberg, 1995). Several eukaryotic activation domains have been reported. These include proline-rich, glutamine-rich and acidic activation domains (Kunzler *et al.*, 1994; Ponticelli *et al.*, 1995; Triezenberg, 1995).

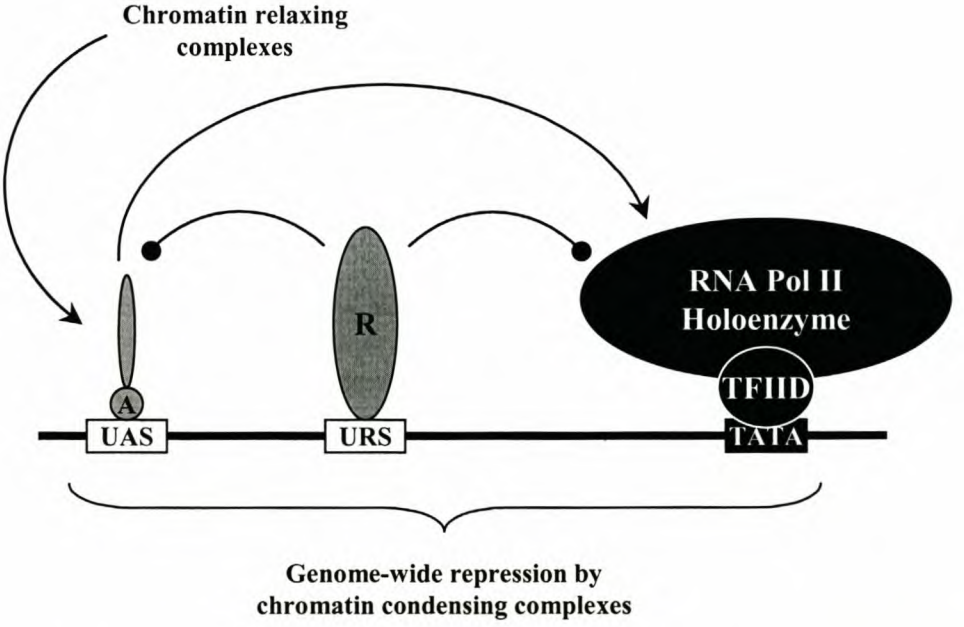

Fig. 2. Function of transcriptional regulators. Arrows indicate activation and balled ends indicate repression.

Most UASs bind a single activator (Struhl, 1989; 1995). Activators can function individually by stimulating the expression of specific gene families or metabolic pathways, such as the coordinate effect of Gcn4p on the transcription of the various target genes in the amino acid biosynthetic genes during starvation (Hinnebusch, 1992). On the other hand, enhancers bind multiple activators to form enhanceosomes. These

are unique enhancer-transcription factor complexes that assemble in response to specific environmental signal(s) and allow the integration of multiple transcriptional activation signals into one combinatorial transcription response (Lee and Young, 2000; Merika and Thanos, 2001). These coordinate and combinatorial activator functions are generally gene-specific. In contrast, some activators have a global function in transcriptional regulation by affecting the expression of various unrelated genes, such as Rap1p and Abf1p (Struhl, 1995).

Pathway-specific activators are generally present at a lower concentration in the cell, in contrast to the general activators that are much more abundant (Struhl, 1995). Pathway-specific activators are major targets of various signal transduction cascades to activate the transcription of various/specific genes in response to environmental conditions. Many activators are constitutively expressed and present in the cell, but are latent until a specific environmental signal activates their function to allow the activated transcription of target genes (Kornberg, 1999). Some pathway-specific activators respond specifically to a compound that induces the expression of genes in the pathway. One such example is Dal82p, a transcription-inducing protein in *S. cerevisiae*, which is required for the induction of the allantoin genes in response to allophanate, the inducer and last intermediate of the allantoin degradative pathway. Dal82p specifically binds the UIS_{ALL} mentioned previously (Dorrington and Cooper, 1993; Olive *et al.*, 1991)

An activator protein enhances transcription by stimulating various steps during this process. It aids in preparing the template, chromatin, for transcription. It does so by recruiting a variety of chromatin altering complexes (discussed later) to the promoter, which will allow access of the transcription machinery to the DNA (Fig. 2) (Cosma *et al.*, 1999; Ikeda *et al.*, 1999; Yudkovsky *et al.*, 1999). Furthermore, it directly interacts with the transcription machinery, thereby recruiting it to the promoter to facilitate transcriptional initiation (Ptashne and Gann, 1997). Following initiation, some activators influence the activity of the transcription apparatus and can increase the overall elongation rate of Pol II by stimulating the rate of promoter escape and/or Pol II processivity (Brown *et al.*, 1998; Yankulov *et al.*, 1994). Some activators can reinitiate transcription, thereby allowing multiple rounds of transcription of a single gene (Struhl, 1996; Zawel *et al.*, 1995).

Repressors

Repression of Pol II transcription is facilitated by two main groups of proteins. Proteins that inhibit the expression of genes in a general manner include chromatin-related proteins (discussed later) and general transcriptional repressors. Gene-specific repressors are used to repress the expression of functionally related genes. Chromatin-related repression results in the genome-wide repression of Pol II transcription, while general and gene-specific repressors function at the promoter level to inhibit transcription (Fig. 2) (Hampsey, 1998; Kornberg, 1999; Lee and Young, 2000).

Gene-specific repressors. A gene-specific repressor affects families of genes and functions by limiting the ability of gene-specific activators to function. It does so either by opposing the activity of DNA-bound activators or by preventing the activators to bind DNA. Repressors can compete with activators for binding to the same DNA-binding element, e.g. the repressor Acr1p binds to the same site as the ATF/CREB activator to repress ATF/CREB-dependent transcription (Vincent and Struhl, 1992). The outcome of this competition will determine the level of expression of the specific gene. In addition, some URS-repressor complexes can recruit chromatin-modifying complexes that condense chromatin, thereby preventing access of activators to DNA. The URS1-Ume6p complex recruits the Sin3-Rpd3 deacetylase complex to repress transcription (Kadosh and Struhl, 1997). These mechanisms prevent the binding of activators to their target elements.

URS-repressor complexes located between UAS-activator complexes and the core transcription machinery provide a steric hindrance that prevents the interaction of the activator with the transcription machinery (Brent and Ptashne, 1984). A repressor can bind the activation domain of an activator in a DNA-independent manner to prevent its interaction with the core transcription machinery. For example, Gal80p, a repressor, binds and masks the activation domain of the Gal4p activator to inhibit its function (Ma and Ptashne, 1987).

General repressors. A general repressor inhibits the expression of many functionally unrelated genes. It does so by inhibiting the core transcription machinery, thereby affecting transcription from promoters in general (Hampsey, 1998; Lee and Young, 2000; Struhl, 1995). Such repressors include Mot1p, the Ydr1-Bur6 complex, and the

Ccr4-Not protein complex. Many of these repressors are, surprisingly, also involved in the transcriptional activation of some genes.

Mot1p is an ATPase that interacts with TBP and dissociates it from the TATAA element in an ATP-dependent manner (Adamkewicz *et al.*, 2000; Auble *et al.*, 1994; Auble and Steggerda, 1999). It therefore represses transcription by preventing the formation of the PIC. This repression is overcome by TFIIA, and to a lesser extent by TFIIB. These proteins compete with Mot1p for binding to the TBP. TFIIA therefore acts to stabilize the TBP-TATAA complex (Auble *et al.*, 1994).

The Ydr1-Bur6 complex, the yeast homologue of human NC2 (Dr1-DRAP), is a heterodimer of the Ydr1-Bur6 complex and acts as a global negative regulator that is essential for yeast viability (Gadbois *et al.*, 1997; Goppelt and Meisterernst, 1996). It binds the promoter-bound TBP to prevent its interaction with TFIIA and TFIIB and therefore inhibits PIC formation (Goppelt *et al.*, 1996).

The *HIS3* gene contains two TATAA elements, T_R and T_C, which bind TBP with high and low affinities, respectively (Ponticelli and Struhl, 1990). Mot1p dissociates the high-affinity TBP-T_R complex of *HIS3* and increases the association of TBP to T_C, thereby increasing transcription from this TATAA element (Collart, 1996). In addition, the expression of *GAL1* and *GAL10* decreases in both the *bur6* and *mot1* mutant strains (Prelich, 1997). These observations show that both these general regulators are involved in transcriptional activation and repression of some target genes (Collart, 1996; Prelich, 1997).

The five Not proteins (Not1p-Not5p) are members of the large global transcription regulator Ccr4-Not. These proteins are needed for general transcriptional repression as loss-of-function mutations increase the transcription of many unrelated genes (Collart and Struhl, 1994; Oberholzer and Collart, 1998). Not1p is central to the function of this complex (Maillet *et al.*, 2000). It associates with TBP (Lee *et al.*, 1998), inhibits the TBP-Spt3p interaction that support transcription (Collart, 1996), and mutation of *NOT1* suppresses the transcriptional defects of *rpb2* and *srb4* mutation (Lee *et al.*, 1998). Srb4p is an essential member of the Pol II holoenzyme and crucial for transcription in general (discussed later). Ccr4p and Caf1p are members of the Ccr4-Not complex needed for the transcriptional activation of many genes (Denis and Malvar, 1990). The

Ccr4-Not complex is therefore involved in transcriptional activation as well as repression.

Co-regulators

Despite the large number of DNA-binding transcriptional regulators in eukaryotes, a variety of regulatory proteins exist that exercise their effect on transcription without binding DNA directly or in a sequence-specific manner. These regulators are called co-regulators and can be classified as either co-activators (Bjorklund and Kim, 1996; Hampsey, 1998; Verrijzer and Tjian, 1996) or co-repressors (Jiang and Stillman, 1992; Keleher *et al.*, 1992; Spector *et al.*, 1997; Tzamarias and Struhl, 1994) according to their function.

Co-activators, also known as adaptors or mediators (Bjorklund and Kim, 1996; Hampsey, 1998; Verrijzer and Tjian, 1996), stimulate transcription either by providing a link between transcriptional activators and the core transcription machinery or by increasing access of the core transcription machinery to the promoter DNA. SRB/Mediator, TAFs, and TFIIA enable the interaction of activators with the core transcription machinery, while some chromatin modifying and remodeling complexes (discussed later) render promoters more accessible for transcription (Fig. 3A).

On the other hand, co-repressors are required to inhibit transcription and do so by either inactivating activators or inhibiting the core transcription machinery or both (Fig. 3B). Promoter-specific co-repressors include the Ssn6-Tup1 and Hir complexes (Keleher *et al.*, 1992; Spector *et al.*, 1997; Tzamarias and Struhl, 1994). Chromatin-modifying complexes that condense DNA, such as the SIR and Sin3-Rpd3 complexes (discussed later), limit the access of the transcription machinery to the promoter DNA and consequently repress transcription on a genome-wide scale (Deckert and Struhl, 2001; Donze *et al.*, 1999; Hecht *et al.*, 1996; Triolo and Sternglanz, 1996). The general transcription repressors that negatively regulate TBP function are also regarded as co-repressors.

SRB/Mediator complex. The SRB/Mediator complex is tightly associated with the Rpb1p CTD (Myers *et al.*, 1998) and impacts on various aspects of Pol II transcription. It is required for basal transcription, activation, repression and stimulates TFIIH function and CTD phosphorylation, which, collectively, influence promoter clearance (Bjorklund and Kim, 1996; Kim *et al.*, 1994; Koleske and Young, 1994; Usheva *et al.*,

1992; Valay *et al.*, 1995). Complexes similar to SRB/Mediator have been identified in higher eukaryotic organisms (Berk, 1999; Rachez and Freedman, 2001).

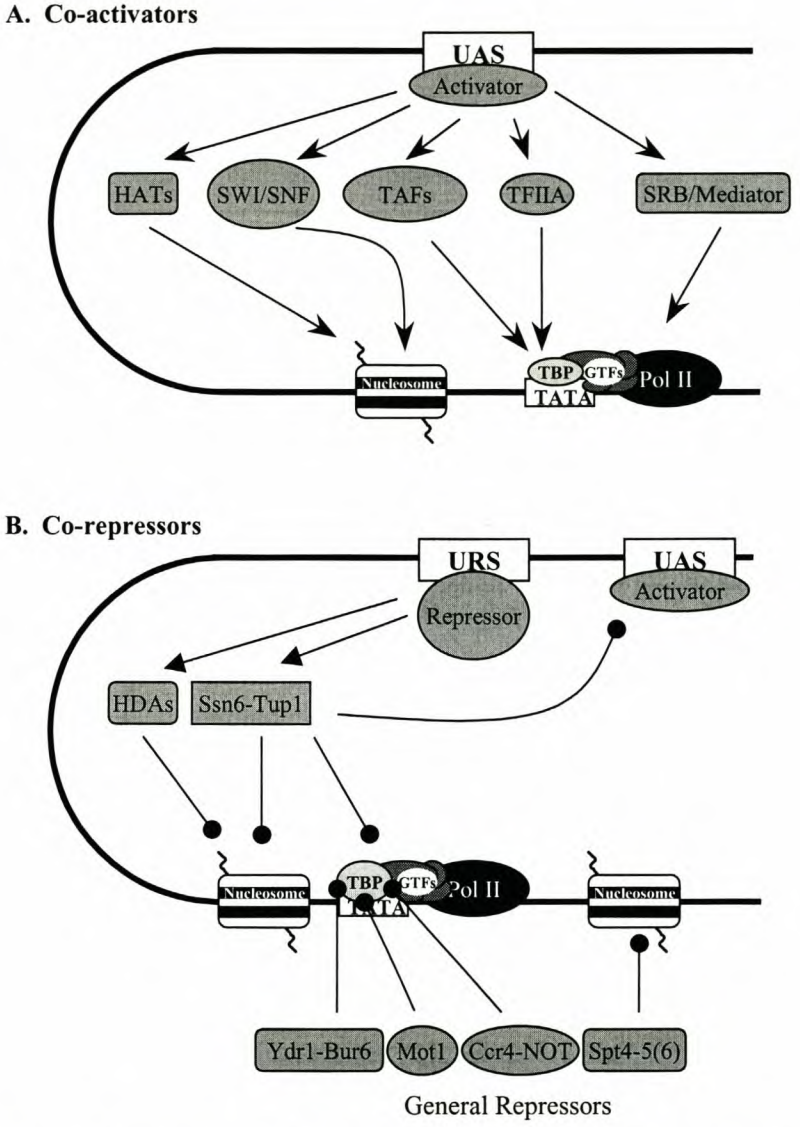

Fig. 3. Function of co-regulators. A) The activating effect of co-activators, and B) the repressing effect of co-repressors (adapted from Hampsey, 1998). Arrows indicate activation and balled ends indicate inhibition.

The SRB/Mediator complex is composed of more than 20 proteins. These include the Ssn-Srb family of proteins, the Med proteins, and a series of previously identified transcriptional regulatory proteins such as Gal11p, Rgr1p, Sin4p, Hrs1p and Rox3p (Han *et al.*, 1999; Hengartner *et al.*, 1995; Kim *et al.*, 1994; Kuchin *et al.*, 1995; Liao *et al.*, 1995; Myers *et al.*, 1998; Thompson *et al.*, 1993). Components of the SRB/Mediator are separated into two stable sub-complexes: the Srb4 and Rgr1 sub-complexes (Lee and Kim, 1998).

Different forms of the SRB/Mediator exist. Complexes either containing (Hengartner *et al.*, 1998) or lacking (Myers *et al.*, 1998) the Srb8-11 proteins have been isolated. Also, components of the SWI-SNF chromatin-remodeling complex have been identified as subunits of the SRB/Mediator (Wilson *et al.*, 1996), while others have reported the absence of these proteins from the SRB/Mediator complex (Myers *et al.*, 1998).

Although the SRB/Mediator is mainly present as a component of the holoenzyme, it also exists as a free complex (Kim *et al.*, 1994) and Svejstrup *et al.* (1997) proposed a model of SRB/Mediator recycling. According to this model SRB/Mediator enters the PIC as a component of the holoenzyme. Following phosphorylation of the CTD during promoter clearance, the SRB/Mediator is released, as it is not associated with elongating Pol II. Free SRB/Mediator is then available to enter a next holoenzyme complex with Pol II.

Srb4 sub-complex. The Srb4 complex contains the genetically dominant Srb proteins (Srb2p, Srb4p, Srb5p, and Srb6p), Med6p and Rox3p (Lee and Kim, 1998). Srb4p is essential for the global transcription of most Pol II genes as the *srb4*^{ts} mutant has severe transcriptional defects at non-permissive temperatures (Holstege *et al.*, 1998; Thompson and Young, 1995). Ydr1-Bur6 and *NOT1* loss-of-function mutants suppress this defect (Gadbois *et al.*, 1997; Lee *et al.*, 1998). Ydr1-Bur6 and the Not complex, of which Not1p is a component, are general repressors in yeast (Collart and Struhl, 1994; Goppelt and Meisterernst, 1996). Collectively these observations indicate that the Srb4 sub-complex enables the holoenzyme to overcome the general negative repression imposed by negative regulators to ultimately form a PIC (Lee *et al.*, 1998). In addition, Srb4p showed weak interaction with Gal4p, the activator needed for activation of the *GAL* genes in response to galactose (Koh *et al.*, 1998).

Rgr1 sub-complex. The remaining SRB/Mediator components form the Rgr1 sub-complex and primarily function by receiving and relaying activator-specific responses via the SRB/Mediator to the transcription machinery (Han *et al.*, 1999; Lee and Kim, 1998). This sub-complex contains the Gal11 module (Gal11p, Sin4p, and Hrs1p) (Li *et al.*, 1995). The gene–specific activator Gal4p specifically interacts with Gal11p (Lee *et al.*, 1999). Two additional components of the Rgr1 sub-complex are targets for specific activators. Med9p and Med10p mediate transcriptional activation signals from Bas1p/Bas2p and Gcn4p, respectively, to activate *HIS4* transcription in amino acid

starvation conditions. Rap1p enhances the positive effects of Bas1p/Bas2p and Gcn4p on *HIS4* transcription under these conditions (Devlin *et al.*, 1991), but does so by interacting with Gal11p (Han *et al.*, 1999). Thus, different transcriptional activators can have requirements for specific components of the Rgr1 sub-complex to exert their stimulatory effects on a specific target gene.

Dual function of the SRB/Mediator. In addition to the role of the SRB/Mediator in transcriptional activation, this complex is also required for the repression of various genes. The Srb8-11 proteins are closely associated with the CTD of Rpb1p and function to repress the transcription of some Pol II genes (Hengartner *et al.*, 1998; Liao *et al.*, 1995). Srb10p is a CTD kinase that negatively regulates Pol II transcription under rich growth conditions (Holstege *et al.*, 1998). It forms a cyclin-kinase pair with Srb11p that prematurely phosphorylates the CTD of Rpb1p in rich nutrient conditions (Hengartner *et al.*, 1998). Phosphorylated CTD cannot be incorporated into a PIC and transcription is consequently prevented.

Rgr1p and Sin4p are components of the SRB/Mediator (Li *et al.*, 1995) and have previously been identified as regulators required for transcriptional activation and repression (Jiang *et al.*, 1995). The transcriptional activation, maximal induction and glucose-dependent repression of the *GAL* genes are dependent on Sin4p and Rgr1p (Chen *et al.*, 1993; Fassler *et al.*, 1991; Sakai *et al.*, 1990). This apparent contradiction in function is explained by the presence of these proteins in the holoenzyme. Regulators required for activation, induction or repression could interact with Sin4p and Rgr1p in the SRB/Mediator complex to consequently relay its transcription signal to the general transcription machinery (Li *et al.*, 1995). Collectively these observations indicate that SRB/Mediator can function to stimulate or repress Pol II transcription.

TAF_{II}s. In addition to their role in start site selection, TAFs function as co-activators of transcription (Burley and Roeder, 1996; Verrijzer and Tjian, 1996). Certain TAFs, like $TAF_{II}90$, bind GTFs, while others, like $TAF_{II}40$ and $TAF_{II}60$, directly interact with transcriptional activators (Chen *et al.*, 1994; Thut *et al.*, 1995). Direct evidence of TAF-function as co-activators comes from $TAF_{II}30$. This protein is a component of the Pol II holoenzyme, and identical to both Tfg3p, a component of TFIIF, and Swi29p, a subunit of the SWI-SNF coactivator complex (Cairns *et al.*, 1996a; Henry *et al.*, 1994). These observations led to the general model in which TAFs function to relay the message/function of activators to the core transcription machinery.

However, the absence of TAF_{II}s from yeast cells did not obviate transcriptional activation (Walker *et al.*, 1996). It was consequently shown that TAF_{II}s are required for the transcriptional regulation of only a subset of genes (e.g. cell cycle progression) and not for general transcriptional regulation (Apone *et al.*, 1996). TAFs therefore have a limited requirement for transcriptional regulation by Pol II.

Ssn6-Tup1 complex. The Ssn6-Tup1 complex, a conserved co-repressor, efficiently represses the transcription of various eukaryotic genes (Carlson, 1997; DeRisi *et al.*, 1997; Fisher and Caudy, 1998; Redd *et al.*, 1996). It has an elongated conformation and consists of one Ssn6 protein and four Tup1 proteins (Redd *et al.*, 1997; Varanasi *et al.*, 1996).

Ssn6-Tup1 has to be recruited to the promoter by DNA-binding regulators to assert its repressive effect on transcription. It binds promoter-specific DNA-binding repressors such as the α2-Mcm1 repressor at **a**-cell-specific genes (Keleher *et al.*, 1992; Komachi *et al.*, 1994) and Mig1p at glucose-repressed genes (Treitel and Carlson, 1995; Tzamarias and Struhl, 1995). Three different mechanisms of repression have been proposed for Ssn6-Tup1. Firstly, it can prevent the activator from functioning. DNA-bound Gal4p is strongly repressed by Ssn6-Tup1. This indicates that it is not the binding of activators, but rather their ability to activate that can be regulated by Ssn6-Tup1 (Redd *et al.*, 1996). Secondly, it directly interacts with the PIC to prevent transcriptional initiation (Tzamarias and Struhl, 1994). Several genetic screens have identified Srb8-11p, Rgr1p, Sin4p and Rox3p as proteins that affect Ssn6-Tup1 repression (Carlson, 1997; Myer and Young, 1998). These proteins are all members of the SRB/Mediator complex and imply that Ssn6-Tup1 interacts with and inhibits the Pol II holoenzyme. Lastly, Ssn6-Tup1 promotes the formation of transcriptionally repressive chromatin. It interacts with components of chromatin and its repressive effect is partially relieved in strains lacking histone deacetylase activity (Edmondson *et al.*, 1996).

The relief of Ssn6-Tup1 mediated repression is targeted at the DNA-binding repressors that recruit it to the promoter. Mig1p is hyperphosphorylated and exported from the nucleus when the yeast encounters low concentrations of glucose. Ssn6-Tup1 is consequently not present at the promoters of glucose-repressed genes, repression is lifted and active transcription follows (De Vit *et al.*, 1997; Ostling and Ronne, 1998). A direct mechanism of Ssn6-Tup1 inactivation has not been found to date.

How are the regulators regulated?

The influence of transcription factors and chromatin on gene expression has been discussed. However, other factors can also influence regulated gene expression. Yeast is able to regulate the concentration and activities of various transcription factors in response to environmental conditions. Mechanisms that enable this regulation include controlled protein synthesis, post-translational modification, small molecule regulation, nuclear localization or degradation.

Controlled protein concentration. The presence or absence of various transcription factors is dependent on environmental conditions. The synthesis of these proteins can be controlled at the level of transcription or translation. The transcription of *GAT1*, which encodes an activator of nitrogen-regulated genes, is only activated in limiting nitrogen conditions (Coffman *et al.*, 1996). Gcn4p is a transcriptional activator of many genes encoding amino acid biosynthetic enzymes. The synthesis of this activator is controlled by a complex translational control mechanism, which ensures the presence of this activator only in amino acid starvation and nutritional down shift conditions (Dever *et al.*, 1992; Mueller and Hinnebusch, 1986). In addition, Gcn4p is also subject to controlled degradation as its concentration is regulated by ubiquitin-mediated proteolysis (Chi *et al.*, 2001; Kornitzer *et al.*, 1994). Ubiquitin-mediated proteolysis and transcriptional activation domains of activators overlap. Thus, one domain (DAD – destruction and activation domain) is responsible for the function and destruction of the activator. This mechanism allows i) the rapid reprogramming of transcription patterns, ii) regulated transcriptional activation by any one transcriptional activator, and/or iii) prevents the accumulation of excess transcriptional activator that could lead to "squelching" of the basal machinery (Salghetti *et al.*, 2000).

Small molecule regulation. Several transcription factors are dependent on small molecules for their function. Hap1p activates the transcription of many genes in response to oxygen. Heme binds this protein and unmasks its DNA-binding domain, thereby allowing the activator to function (Zhang and Guarente, 1995). In contrast, α-isopropylmalate is an intermediate of the leucine synthetic pathway and is required by Leu3p for transcriptional activation, not for DNA-binding (Sze *et al.*, 1992).

Post-translational modification. Phosphorylation represents the major mechanism of transcription factor regulation. Kinases act in response to environmental stimuli to

phosphorylate and regulate the activity of these proteins. Mating pheromones stimulate the rapid phosphorylation of the Ste12p activation domain with the consequent increase in the transcriptional activity of the protein (Song *et al.*, 1991). In contrast, Pho80p/Pho85p phosphorylated Pho4p to repress its ability to activate transcription in high concentrations of phosphate (Kaffman *et al.*, 1994). In low concentrations of phosphate, Pho81p inhibits Pho4p phosphorylation and allows it to activate its target genes (Schneider *et al.*, 1994). Thus, phosphorylation can have a positive or negative effect on transcription.

Nuclear localization. Activators can only activate transcription when present in the nucleus. Environmental conditions control the nuclear translocation of many transcriptional activators. A signaling cascade of proteins senses the environment and responds by activating a cascade of proteins. Its ultimate function is to modify the activator to retain it in the cytoplasm or to translocate it to the nucleus. Gln3p is an activator that activates the transcription of nitrogen-regulated genes in response to poor nitrogen conditions. In rich nitrogen conditions, Gln3p is phosphorylated, bound to Ure2p and retained in the cytoplasm. In poor nitrogen conditions, Gln3p is dephosphorylated and translocates to the nucleus to activate the transcription of its target genes (Beck and Hall, 1999; Bertram *et al.*, 2000).

Architectural transcription factors. In many instances proteins or protein complexes need to interact to regulate Pol II transcription. Architectural proteins bend DNA into conformations that favor the interaction of distant complexes to perform this regulation (Werner and Burley, 1997). Architectural factors contain a DNA binding domain similar to that first identified in mammalian high-mobility-group 1 and 2 (HMG-1 and -2) architectural proteins (Bustin and Reeves, 1996). The mammalian HMG-1 proteins are very abundant chromatin-binding proteins (Bustin, 1999) implicated in transcriptional activation and repression, nucleosome assembly and chromatin organization (Ge and Roeder, 1994; Nightingale *et al.*, 1996; Shykind *et al.*, 1995). Several yeast proteins, including Rox1p, Sin1p, Nhp6Ap and Nhp6Bp contain these domains. Rox1p binds DNA in a sequence-specific manner (Deckert *et al.*, 1995), while Sin1p, Nhp6Ap and Nhp6Bp bind DNA non-specifically (Kruger and Herskowitz, 1991; Paull and Johnson, 1995).

Nhp6Ap and Nhp6Bp are involved in DNA bending and enhance the assembly of multi-component protein-DNA complexes, including pre-initiation complexes (Paull *et*

al., 1996; Paull and Johnson, 1995). These proteins affect the expression of some genes positively and others negatively and influence Pol II transcription at a gene-specific rather than chromosomal domain level (Moreira and Holmberg, 2000; Paull and Johnson, 1995).

THE TEMPLATE FOR TRANSCRIPTION - CHROMATIN

The large genomes of eukaryotes are wrapped into highly condensed protein-DNA structures, called chromatin, to enable packaging into the limited space of the nucleus. This condensation poses an accessibility problem for proteins required to perform several DNA-related processes including transcription, replication, recombination and DNA repair. Chromatin is integral to the transcriptional regulation of genes as it prevents unsolicited transcription by limiting access of the general transcription machinery or regulatory proteins to regulatory DNA sequences (Gregory, 2001). The DNA-binding proteins only gain access to the condensed DNA once the chromatin structure is remodeled to a more transcriptionally permissive state. Multisubunit complexes perform chromatin remodeling in a covalent or non-covalent manner. In many cases the predominant role of transcriptional activators is to recruit chromatin remodelers that ultimately facilitate Pol II access to its chromatin template (Gregory, 2001; Grunstein, 1997b; Wolffe and Guschin, 2000; Workman and Kingston, 1998).

Chromatin Structure

The condensation of nuclear DNA into chromatin involves a hierarchical system of organization. The first and second levels of condensation involve the formation of nucleosomes (beads-on-a-string) and then the 30 nm fiber, respectively. The third and higher levels of condensation involve the compaction of the 30 nm fiber into increasingly constrained loop domains (Donze *et al.*, 1999; Widom, 1998). The ability to activate transcription decreases as the higher-order structure of the chromatin increases (Bi and Broach, 2001; Braunstein *et al.*, 1996).

Nucleosomes

Chromatin consists of an array of nucleosomes interspersed with linker DNA to give rise to its familiar beads-on-string structure. In yeast, a nucleosome consists of

DNA wrapped around an octamer of four core histone proteins, H2A, H2B, H3, and H4. The assembly of a stable nucleosome is dependent on various histone-histone and histone-DNA interactions. The initial $(H3/H4)_2$ tetramer complexes with 120 bp DNA. H2A/H2B heterodimers bind to the tetramer-DNA complex via H2B-H4 interactions and extends the DNA wrapped in the nucleosome to 147 bp (Hayes *et al.*, 1990, 1991b; Luger *et al.*, 1997). Histone-DNA interactions distort the structure of nucleosomal DNA to fairly similar conformations, independent of the DNA sequence (Hayes *et al.*, 1991a).

In higher eukaryotes, a fifth histone protein, the linker histone (H1), interacts with the nucleosome core (proteins and DNA) and linker DNA separating nucleosomes (Clark and Kimura, 1990; Zhou *et al.*, 1998). These interactions are not essential for nucleosome assembly (Shen *et al.*, 1995), but stabilize nucleosomes and facilitate the folding of chromatin into higher-order structures (Carruthers *et al.*, 1998; Clark and Kimura, 1990).

The amino termini of all four core histones and the carboxyl terminus of H2A contain positively charged "tail" domains that extend from the globular structure of the histone octamer. Removal of these domains does not alter the conformation or correct assembly of individual nucleosomes (Ausio *et al.*, 1989; Dong *et al.*, 1990; Hayes *et al.*, 1991a). However, the specific positioning of nucleosomes at some yeast promoters, which leads to repressed expression of the associated gene, require these tail domains (Fisher-Adams and Grunstein, 1995; Roth *et al.*, 1992). In addition, these tail domains extend beyond the nucleosomal DNA (Wolffe and Hayes, 1999) and is required for internucleosomal interactions when chromatin is condensed into higher-order chromatin (Fletcher and Hansen, 1996). Lastly, several proteins and protein complexes interact with the tail domains. These include transcriptional regulators, such as the repressor Tup1p (Edmondson *et al.*, 1996) and the silent information regulator (SIR) complex (Hecht *et al.*, 1995), chromatin modifying complexes, like SAGA and Sin3-Rpd3 (Grant *et al.*, 1997; Taunton *et al.*, 1996), and chromatin remodeling complexes, such as SWI-SNF (Georgel *et al.*, 1997; Wolffe and Guschin, 2000). These interactions are responsible for the transcriptional regulation of various eukaryotic genes.

Non-histone proteins

Several non-histone chromatin proteins, including the architectural proteins and the gene products of the *SPT*, *SIN* and *SIR* genes, associate with and provide functional diversity to chromatin (Bustin and Reeves, 1996; Bustin, 1999). These proteins can serve as additional targets for the action of regulators that control Pol II transcription.

A subset of *SPT* gene products (Spt2p, Spt4p-6p, Spt11p and Spt12p) play important roles in the assembly, condensation and regulation of the chromatin structure. Besides *SPT11* and *SPT12*, which encode histones H2A and H2B, respectively (Clark-Adams *et al.*, 1988), the other *SPT* genes encode non-histone chromatin proteins. Spt2p/Sin1p is an HMG1-like protein that non-specifically binds DNA (Kruger and Herskowitz, 1991; Roeder *et al.*, 1985). Spt4p, Spt5p and Spt6p form a complex that affects transcription in yeast (Clark-Adams and Winston, 1987; Malone *et al.*, 1993; Swanson *et al.*, 1991). Spt6p interacts with histones and, with a limited dependence on Spt4p and Spt5p, assembles and reorganizes nucleosomes (Bortvin and Winston, 1996).

Mutations of the *SIN* (*SIN1-4*) genes suppress the negative effects of *swi* mutations on gene expression (Nasmyth *et al.*, 1987; Sternberg *et al.*, 1987). Besides *SIN2/HHT1*, which encodes H3 (Kruger *et al.*, 1995), the remaining *SIN* genes encode non-histone proteins. *SIN1* and *SPT2* are allelic and their products share characteristics of the mammalian HMG-1 proteins (Kruger and Herskowitz, 1991). Sin1p is an abundant chromatin component that binds DNA non-specifically and has a repressing effect on transcription. It binds and stabilizes nucleosomes, thereby having a negative effect on transcription. However, it is also needed for transcriptional activation mediated by the SWI-SNF chromatin-remodeling complex (discussed later) (Pérez-Martín and Johnson, 1998). Sin3p is a negative transcriptional regulator and an integral subunit of the Sin3-Rpd3 histone deacetylase complex (discussed later) (Bernstein *et al.*, 2000; Kadosh and Struhl, 1997). Lastly, Sin4p is a regulatory protein that represses or stimulates transcription (Chen *et al.*, 1993; Covitz *et al.*, 1994; Li *et al.*, 1995). It is involved in chromatin organization and structure (Jiang and Stillman, 1992; Macatee *et al.*, 1997), and associates with the Pol II holoenzyme complex (Li *et al.*, 1995; Myers *et al.*, 1998) and SAGA complex (Yu *et al.*, 2000).

NHP6A and *NHP6B* encode the yeast Nhp6Ap/6Bp architectural proteins that belong to the HMG-1 protein family (Kruger and Herskowitz, 1991). Nhp6Ap and

Nhp6Bp are needed for the chromatin-mediated regulation *HO* gene expression (Yu *et al.*, 2000).

Chromatin-mediated Transcriptional Regulation

The repressive effect of chromatin on basal transcription has to be overcome when the transcription of condensed DNA is required. Eukaryotes employ a variety of chromatin altering activities to transit from transcriptionally repressive to transcriptionally permissive DNA (Pérez-Martín, 1999). These activities can be divided into two general groups. The first group uses the energy of ATP hydrolysis to remodel chromatin non-covalently by disrupting the histone-DNA interactions within chromatin. The conserved SWI-SNF complex is a member of this group (Workman and Kingston, 1998). The second group covalently modifies the tails of core histones, thereby affecting histone-histone and histone-DNA interactions. SAGA-related co-activators and Sin3-Rpd3-related co-repressors are members of this group (Grunstein, 1997b; Workman and Kingston, 1998).

Chromosomal domains

The local chromatin structure of promoters has direct implications for transcription. The genomes of eukaryotes are arranged in functional chromosomal domains, called euchromatin or heterochromatin that either potentiate or repress gene expression, respectively (Elgin, 1996; Bi and Broach, 2001). Heterochromatin represses or silences the genes in its structure due to highly condensed chromatin. Heterochromatic domains, such as those present in the telomeres and the *HML* and *HMR* loci of yeast, originate at *cis*-acting elements, known as silencers (Kamakaka, 1997; Laurenson and Rine, 1992). The SIR proteins are required for the transcriptional silencing of telomeric DNA and the silent mating type loci (Aparicio *et al.*, 1991). Sir1p is needed to establish silent chromatin as it plays an integral part in recruiting the SIR complex to silencer elements (Triolo and Sternglanz, 1996). Sir2p is an NAD-dependent histone deacetylase (Imai *et al.*, 2000) that produces hypoacetylated histones, which are associated with transcriptionally silent chromatin (Braunstein *et al.*, 1993; 1996). Sir3p and Sir4p homo- and heterodimerize, complexes with Sir2p and interact with the core histone tails (Hecht *et al.*, 1995; 1996; Strahl-Bolsinger *et al.*, 1997) to form the SIR complex (Hecht *et al.*, 1996). These silencer elements contain multiple binding sites for transcriptional

activators such as Abf1p and Rap1p, and autonomously replicating sequences (ARSs) for the Origin of Replication Complex (ORC) (Fig. 4). These proteins bind silencers and recruit the SIR complex to chromatin (Loo and Rine, 1994; 1995). The SIR complexes polymerize bidirectionally via Sir3p, thereby spreading the silencing effect along the chromatin (Donze *et al.*, 1999; Hecht *et al.*, 1996; Triolo and Sternglanz, 1996). Gene non-specific silencing also occurs near the telomeric regions of chromosomes and within rDNA repeats in yeast (Gottschling *et al.*, 1990; Smith and Boeke, 1997).

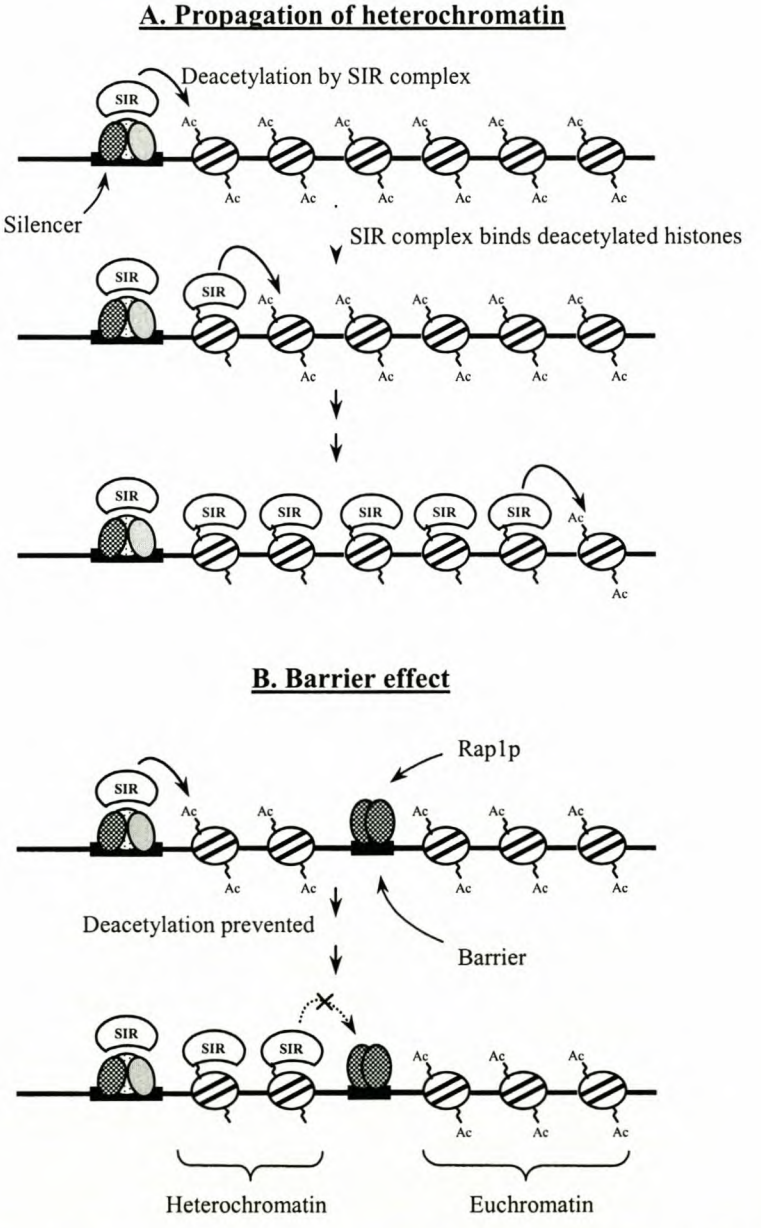

Fig. 4. Chromosomal domains. A) The formation of heterochromatin, and b) the barrier effect that prevents the spread of heterochromatin.

In contrast, euchromatic domains (euchromatin) have a more relaxed chromatin structure, which arises from the disruption of higher-order condensation of chromatin with the consequent increased access of the transcription machinery to its target sites. Euchromatin is consequently more permissive to transcription (Bi and Broach, 2001; Braunstein *et al.*, 1996). Factors that contribute and maintain the open state of euchromatin include the actions at a specific promoter and the activities of enhancers and LCRs (Bulger and Groudine, 1999).

Heterochromatin and euchromatin are interspersed along chromosomes, thereby juxtaposing highly repressive and potentially active transcriptional regions. *S. cerevisiae* and higher eukaryotes contain DNA elements, known as insulators (or boundary elements) and barriers, that demarcate these regions. Insulators are thought to be neutral elements as they eliminate the positive influence of enhancers on transcription when they are located between an enhancer and its target promoter (Geyer and Corces, 1992; Kellum and Schedl; 1992; Sun and Elgin, 1999).

Barrier elements function by preventing the spread of silent chromatin (Donze *et al.*, 1999; Sun and Elgin, 1999). Common characteristics of all barriers include the local exclusion of nucleosomes and chromatin remodeling (Fig. 4). A nucleosome-free gap can be created within the array of nucleosomes. As the SIR complex is proposed to spread heterochromatin by sequentially interacting with adjacent nucleosomes, such a gap would provide a physical obstacle in the formation of silent chromatin. Barriers therefore have a position-dependent effect on a specific promoter, as they will only prevent the silencing of a specific promoter when located between the silencer and the specific promoter. Two examples of passive barrier function include the Rap1p-mediated disruption of chromatin formation (Chasman *et al.*, 1990) and the active transcription of the *HMR* $tRNA^{Thr}$ gene by the Pol III complex to prevent nucleosome positioning in this region (Donze *et al.*, 1999; Donze and Kamakaka, 2001; Morse *et al.*, 1992; Yu and Morse, 1999). A second model for barrier function implies competing chromatin-modifying activities (Donze and Kamakaka, 2001). Sir2p-mediated deacetylation is pivotal in the formation of heterochromatin (Imai *et al.*, 2000). Donze and Kamakaka (2001) reported a loss of barrier activity in a *S. cerevisiae* strain devoid of the Gcn5p or Sas2p histone acetyltransferases. The authors propose that Sir2p-mediated deacetylation starts at a silencer element and proceed along the chromatin until it encounters a barrier element. The stable recruitment of one or more

acetyltransferase activity to the barrier element competes with Sir2p for the histone tail as substrate. This acetylation and deacetylation of the core histone tails prevent the spread of silent chromatin and represent active barrier function.

Chromatin modification

The tail domains of the core histones are targets for several covalent modifications that change their charge and/or conformation. The modifications include acetylation/deacetylation, phosphorylation, ubiquitination, and methylation (Strahl and Allis, 2000; Workman and Kingston, 1998).

Acetylation/Deacetylation. Several conserved lysine residues in the tail domains of core histones are targets for reversible acetylation. Histone acetyltransferases (HATs), like Gcn5p and Esa1p in yeast, catalyze the acetylation of these lysines by using acetyl-CoA as a donor molecule (Allard *et al.*, 1999; Brownell *et al.*, 1996; Grunstein, 1997a). The nucleosomes in many transcriptionally active yeast promoters are hyperacetylated in a Gcn5p- and/or Esa1p-dependent manner, thereby linking transcriptional activation and the hyperacetylation of chromatin (Allard *et al.*, 1999; Brownell *et al.*, 1996; Galarneau *et al.*, 2000; Kuo *et al.*, 1996; Reid *et al.*, 2000). In addition, HAT activities, like the human $TAF_{II}250$ and yeast $TAF_{II}130$, are associated with the TFIID complex (Geisberg and Struhl, 2000; Lee *et al.*, 2000; Mizzen *et al.*, 1996).

Hyperacetylated histones are found in transcriptionally active chromatin (Hebbes *et al.*, 1994), while deacetylated chromatin is normally associated with transcriptionally inert chromatin (Braunstein *et al.*, 1993). Acetylation neutralizes the positive charge of the core histone tail, thereby disrupting the histone-histone and histone-linker DNA interactions (Grunstein, 1997b; Luger and Richmond, 1998). Acetylated histones also wrap nucleosomal DNA less tightly. The higher-order condensed structure of chromatin is consequently compromised to yield transcriptionally competent chromatin (Bauer *et al.*, 1994; Krajewski and Becker, 1998).

Conversely, these acetyl groups are removed from the specific lysine residues by histone deacetylases (HDACs) (Grunstein, 1990). A correlation exists between transcriptionally inert and hypoacetylated chromatin as the transcriptional activity of these regions increases when cells are treated with HDAC inhibitors such as trichostatin A (Wong *et al.*, 1998; Yoshida *et al.*, 1990). Several HDACs have been identified in eukaryotes, including the Rpd3p, Hda1p, Hos1p, Hos2p, Hos3p and Sir2p from yeast

(Carmen *et al.*, 1996, 1999; Imai *et al.*, 2000; Rundlett *et al.*, 1996; Taunton *et al.*, 1996).

Hypoacetylated chromatin is generally associated with transcriptional repression. Repression can result from the actions of transcriptional repressors that preferentially bind hypoacetylated nucleosomes. The transcriptional repressor Tup1p interacts with hypoacetylated H3 and H4 (Edmondson *et al.*, 1996; Mukai *et al.*, 1999). Tup1p is also a component of the Ssn6-Tup1 corepressor complex that interacts with Rpd3p and Hos2p (Waterborg, 2000). In addition, deacetylated histone tails enable increased interaction between adjacent nucleosomes. Deacetylation therefore results in an increased ability of chromatin to condense into more compact, transcriptionally inert heterochromatin (Braunstein *et al.*, 1993; Fletcher and Hansen, 1996; Luger *et al.*, 1997).

The nucleosomes in the yeast genome are generally moderately acetylated with each histone tail containing two acetylated lysines on average. In contrast, the nucleosomes of higher eukaryotes are more severely hypoacetylated, giving rise to a much larger percentage of the genome contained in transcriptionally inert heterochromatin when compared to yeast (Waterborg, 2000).

Struhl (1998) proposed three general models according to which HATs and HDACs influence gene expression. Firstly, these two enzyme activities can work non-specifically on a genome-wide level. Thus, gene-specific expression responses would arise from the ability of transcriptional regulators to bind their respective packaged DNA binding sites. Secondly, HATs and HDACs can be selectively targeted to promoters per se, but with little selectivity for a particular gene. Consider the $TAF_{II}130/250$ HAT. It is a component of TFIID and is hence targeted to most, if not all, Pol II promoters. Lastly, these two modifying activities can be recruited to specific promoters by DNA-bound regulators to generate localized chromatin structures that would either enable or prevent transcription. Histones are generally acetylated to facilitate DNA replication, followed by chromatin maturation, which entails extensive deacetylation and chromatin condensation into higher-order structures (Fletcher and Hansen, 1996). This transient state of decondensed chromatin presents an opportunity for transcription factors to access target sites and for chromatin remodeling enzymes to function. Both Gcn5p and Esa1p assist in global acetylation (Allard *et al.*, 1999; Krebs *et al.*, 1999; Reid *et al.*, 2000). Following the S phase, Sin3-Rpd3 deacetylates histones

(Krebs *et al.*, 1999). This balance between acetylation and deacetylation is disrupted at the promoter level by the actions of gene-specific transcription factors.

HAT complexes. Several HAT activities have been identified in yeast and are normally associated with multisubunit complexes, including ADA-Gcn5p, SAGA, NuA3, NuA4, yTFIID, Elongator and the HAT1-complex (Allard *et al.*, 1999; Grant *et al.*, 1997; Lee and Young, 2000; Luger and Richmond, 1998). The Gcn5p HAT is contained in two coactivator complexes: ADA-Gcn5p (Ada1p, Ada2p, Ada3p, and Gcn5p) and SAGA (SPT proteins, ADA proteins and Gcn5p) (Georgakopoulos and Thireos, 1992; Georgakopoulos *et al.*, 1995; Grunstein *et al.*, 1995; Horiuchi *et al.*, 1997; Utley *et al.*, 1998). Esa1p is found in NuA4 (Allard *et al.*, 1999). SAGA, ADA-Gcn5p and NuA3 primarily acetylate histone H3, while NuA4 preferentially acetylates histone H4 (Allard *et al.*, 1999; Grant *et al.*, 1997). These observations confirm that different yeast HATs have different core histone substrate specificities, which have distinct functions in transcriptional regulation (Fisher-Adams and Grunstein, 1995).

SAGA is a >20 subunit protein complex with Gcn5p as the catalytic activity (Kuo *et al.*, 1998; Wang *et al.*, 1998). Gcn5p specifically acetylates histone H3 tail domains (Zhang *et al.*, 1998) and is essential for the transcriptional activation of various Pol II-dependent genes (Kuo *et al.*, 1998; Wang *et al.*, 1998). Two SPT proteins, Spt20p and Spt3p, are required for the SAGA-mediated recruitment of TBP to some Pol II promoters (Dudley *et al.*, 1999b). Spt20p is essential for the SAGA complex integrity (Grant *et al.*, 1997), while Spt3p is a TAF-like component of SAGA (Birck *et al.*, 1998) that interacts with TBP (Eisenmann *et al.*, 1992). Although the *spt3* mutant has significant transcriptional defects, the SAGA complexes isolated from these cells still contain a Gcn5p-dependent HAT activity. Thus, Spt3p affects transcription mediated by SAGA independent of histone acetylation (Dudley *et al.*, 1999a; Sterner *et al.*, 1999). Ada2p interacts with the acidic activation domains of several transcription factors (Barlev *et al.*, 1995). Consistently, SAGA is recruited to promoters by various activators, including Swi5p (Cosma *et al.*, 1999), Gcn4p (Natarajan *et al.*, 1998) and RTG3 (Massari *et al.*, 1999).

SAGA has different functions at different promoters. The role of SAGA at the *GAL1* promoter is largely independent of the Gcn5p-activity and focuses on the interaction between Gal4p and general transcription factors (Dudley *et al.*, 1999b; Koh *et al.*, 1998; Melcher and Johnston, 1995; Wu *et al.*, 1996). In *spt3* and *spt20* mutants

Gal4p binds normally to the *GAL1* promoter, but TBP fails to bind. Transcription is 50-fold lower than in the WT strain. Thus, these proteins are required for the recruitment of TBP to the *GAL1* promoter (Dudley *et al.*, 1999b). SAGA can therefore function as a coactivator by connecting promoter-specific activators with TBP or other general transcription factors (Dudley *et al.*, 1999b; Hampsey, 1998; Hampsey and Reinberg, 1999). In contrast to the Gcn5p-independence of the *GAL1* promoter, the SAGA-mediated binding of Swi4p to the *HO* promoter and the consequent transcriptional activation is heavily Gcn5p-dependent (Cosma *et al.*, 1999). Dudley *et al.* (1999b) propose that the flexibility in SAGA function at different promoters is facilitated by the distinct functions of each of the subunits associated with SAGA.

Certain DNA-bound transcription factors, including regulators and subunits of the basal transcription machinery, specifically interact with and recruit HAT-containing complexes to specific promoters. TAF_{II}s have been associated with the SAGA complex, transcriptional activators, like Swi5p and Gcn4p, recruit SAGA to specific promoters, Ada2p interacts with both TBP and the acidic activation domains of various transcriptional activators, and SAGA functionally interacts with SWI-SNF and SRB/Mediator complexes (Barlev *et al.*, 1995; Cosma *et al.*, 1999; Dudley *et al.*, 1999a; Grant *et al.*, 1998; Grunstein *et al.*, 1995; Roberts and Winston, 1997). These interactions enable gene-specific targeting of the HAT-activity, hyperacetylation of local promoter nucleosomes and the consequent increased access of transcription proteins to nucleosomal DNA (Cosma *et al.*, 1999; Ikeda *et al.*, 1999; Kuo *et al.*, 1998).

Esa1p is an essential protein that provides the critical H4 acetylating activity required for the NuA4 HAT complex to function (Allard *et al.*, 1999; Clarke *et al.*, 1999). NuA4 is recruited by various transcription activators (like Gcn4p) and it stimulates the transcription of chromatin (Galarneau *et al.*, 2000; Ikeda *et al.*, 1999; Reid *et al.*, 2000; Steger *et al.*, 1998; Utley *et al.*, 1998). USF, a transcriptional activator, also preferentially binds to acetylated H4 (Vettesse-Dadey *et al.*, 1996), while the transcriptional repressors Tup1p and Sir3p function *via* deacetylated histone H4 (Edmondson *et al.*, 1996; Hecht *et al.*, 1995). NuA4 therefore functions as a transcriptional coactivator that stimulates transcription by acetylating histone H4 (Allard *et al.*, 1999). Esa1p plays a global role in H4 acetylation (Galarneau *et al.*, 2000). Although H4 acetylation generally decreases in an *esa1* mutant, it was surprisingly that the transcription of many genes is not affected. Specific targeting of

Esa1p to specific promoters is required to affect transcription in the background of non-specific acetylation. Esa1p is preferentially associated with the promoters of Pol II genes encoding ribosomal proteins and/or heat shock proteins *via* the activators Rap1p and Hsf1p, respectively (Reid *et al.*, 2000).

Amongst the other components of the 11-subunit NuA4 complex are the essential proteins Tra1p and Act3p/Arp4p (Allard *et al.*, 1999; Galarneau *et al.*, 2000). Tra1p shares similarity with phosphoinositol-3 kinases, thereby suggesting a possible role for NuA4 in signal transduction (Allard *et al.*, 1999). Tra1p is also found in SAGA and SAGA-related complexes and binds the Gcn4p activation domain (Allard *et al.*, 1999; Grant *et al.*, 1998; Saleh *et al.*, 1998). These observations confirm the interaction of NuA4 with transcriptional activators. Act3p/Arp4p is a nuclear protein that forms ternary complexes with nucleosomes (Galarneau *et al.*, 2000; Jiang and Stillman, 1996). Act3p/Arp4p supposedly enhances the binding of NuA4 to chromatin (Galarneau *et al.*, 2000).

In addition to the disruption of the chromatin structure, acetylated histones can serve as targets for interaction with chromatin remodeling complexes (discussed later) and influence the interactions of specific transcription factors with chromatin. Increased hyperacetylation increases the ability of USF, Gcn4p and TFIIA to bind corresponding nucleosomal DNA-binding sites *in vivo* (Lee *et al.*, 1993; Vettese-Dadey *et al.*, 1996). However, different transcriptional activators determine specific histone acetylation patterns to elicit transcriptional activation. The actions of Gcn4p and the stress-inducible activators (Msn2p & Msn4p) facilitate increased histone acetylation, while Gal4p, Hap4p and Met4p-mediated transcriptional activation is associated with deacetylated histones. Thus, transcriptional activation can also be associated with local acetylation or deacetylation of histones at specific promoters (Deckert and Struhl, 2001).

Activator-dependent recruitment of HAT complexes, like SAGA and NuA4, would result in the local hyperacetylation of promoter histones H3 and H4, respectively. Gcn4p interacts with both SAGA and NuA4 complexes and therefore has the potential to acetylate both H3 and H4 of the target promoter (Natarajan *et al.*, 1999; Utley *et al.*, 1998).

Non-histone acetylation. Histones are not the only targets of acetylases. Non-histone proteins, such as general and promoter-specific transcription factors as well as architectural proteins, can also be acetylated (Gu and Roeder, 1997; Imhof *et al.*, 1997; Munshi *et al.*, 1998). Such acetylations can have a positive (as for p53 and Sin1p acetylation) or negative (as for HMG-1) effect on transcription (Gu and Roeder, 1997; Munshi *et al.*, 1998; Pollard and Peterson, 1997). In addition, the general transcription factors TFIIE and TFIIF are also targets of acetylation (Imhof *et al.*, 1997). Collectively these observations suggest additional roles for HATs and HDACs in regulated gene expression. The possibility therefore exists that the transcriptional activities of some promoters are controlled by acetylation/deacetylation of both histone and non-histone proteins (Ayer, 1999).

HDAC complexes. Like HATs, HDACs are also associated with multisubunit complexes. Two HDAC complexes, HDA and HDB, have been purified from yeast (Carmen *et al*, 1996; Rundlett *et al.*, 1996). Rpd3p, a general regulatory factor and histone deacetylase, and Sin3p, a suppressor of *snf/swi* mutations, interact to form the HDB complex called Sin3-Rpd3 (Kadosh and Struhl, 1997; Kasten *et al.*, 1997; Rundlett *et al.*, 1996; Sternberg *et al.*, 1987; Vidal *et al.*, 1991). These two proteins are functionally highly dependent on each other (Bernstein *et al.*, 2000). The *rpd3* mutant lacking the deacetylase activity, but retaining the ability to form the HDB complex, has a decreased ability to repress transcription of selected genes (Kadosh and Struhl, 1998). Sin3p has the characteristics of a transcriptional repressor as the *sin3* mutant obviates the normal requirement of Gcn5p and the Swi5p transcriptional activator to activate *HO* expression (Pérez-Martín and Johnson, 1998; Sternberg *et al.*, 1987). Rather than attributing the repressing effect of Sin3p to its gene-specific targeting, Peterson and Logie (2000) proposed a more global transcriptional regulatory role for Sin3p due to its function in deacetylating newly formed acetylated chromatin following DNA replication. The *sin3* mutant could leave newly formed chromatin acetylated, ready to support transcriptional activation. In sum, Sin3-Rpd3 is perceived as a transcriptional corepressor that deacetylates chromatin to repress transcription (Kadosh and Struhl, 1998; Peterson and Logie, 2000).

Genome-wide transcriptional profiling of the *rpd3* and *sin3* mutants showed that both proteins function to repress and activate the transcription of the same sets of genes (Bernstein *et al.*, 2000). Rpd3p is needed to activate the transcription of telomeric,

silenced genes by opposing the action of the SIR complex. The SIR complex preferentially binds H4 with lysine 12 acetylated. Rpd3p deacetylates this lysine residue, resulting in the inability of SIR to silence genes. The resulting activation is therefore an indirect effect of Rpd3p deacetylation (Bernstein *et al.*, 2000; Hecht *et al.*, 1995).

The Sin3-Rpd3 corepressor complex can be recruited to DNA in various ways. Ume6p, a DNA-binding protein, represses several yeast promoters in a Rpd3p- and Sin3p-dependent manner. DNA-bound Ume6p recruits Sin3-Rpd3 to deacetylate the local H3 and H4 core histones, with the consequent transcriptional repression (Kadosh and Struhl, 1997). Sin3-Rpd3 also binds acetylated chromatin non-specifically as it deacetylates chromatin in a global manner (Deckert and Struhl, 2001; Krebs *et al.*, 1999).

Phosphorylation. Serine residues in the N-terminal tail domain of histone H3 in mammalian cells are phosphorylated by the Rsk2p protein kinase in response to external stimuli (Mahadevan *et al.*, 1991; Sassone-Corsi *et al.*, 1999). Phosphorylation alters the integrity of the core nucleosome structure, with the consequent disruption of the higher-order chromatin structure and increased transcriptional activation (Chen and Allfrey, 1987). Also, hyperphosphorylation of linker histone H1 alleviates the repressive effects of the linker histone on transcription (Lee and Archer, 1998). Thus, histone phosphorylation in response to environmental signals alters the repressive chromatin structure to produce a transcriptionally competent environment. The mechanism of phosphorylation of core histones in response to environmental stimuli to increase transcription in yeast is poorly understood.

Ubiquitination. The C-terminal tail of H2A is a target for ubiquitination. This modification does not significantly alter the core nucleosomal conformation, but does interfere with the condensation of chromatin into higher-order chromatin by affecting the linker histone function and internucleosomal interactions. Ubiquitinated H2As are especially found in the 5' end of transcriptionally active genes and are proposed to permit access of *trans*-acting factors to target regions in chromatin (Wolffe and Guschin, 2000).

Methylation. Core histones H3 and H4 can also be methylated to different degrees, in addition to the above-mentioned modifications. These methylations occur at

the arginine and lysine residues of H3. Methylation has been linked to transcriptional activation as CARM1, a co-activator-interacting protein, has H3-specific methyltransferase activity. Interestingly, CARM1 also functions in association with HAT-containing and kinase-containing co-activators. These observations prompted the hypothesis that multisubunit complexes containing various histone-modifying activities exist and function in regulated gene expression (Strahl and Allis, 2000).

In sum, these covalent additions to histones generally disrupt the condensed structure of chromatin to allow access of transcription factors and chromatin-altering complexes to the DNA. The combined actions of these proteins result in transcriptional regulation of the target genes.

Chromatin remodeling

In addition to covalent modifications, eukaryotes also employ non-covalent conformational remodeling of chromatin to overcome the transcriptional control imposed by nucleosomes (Kornberg and Lorch, 1999; Workman and Kingston, 1998). ATP-dependent mechanisms disrupt and reconstitute histone-DNA interactions, thereby allowing the mobilization and repositioning of nucleosomes within the chromatin (Kingston and Narlikar, 1999; Wolffe and Guschin, 2000). Ultimately, the transcription machinery and transcriptional regulators have increased access to its respective DNA target sites (Utley *et al.*, 1998).

Various non-covalent chromatin-remodeling complexes have been identified, all of which contain a nucleic acid-dependent ATPase that is essential to perform ATP-dependent remodeling (Côté *et al.*, 1994; Imbalzano *et al.*, 1994). Additional subunits in each complex determine the specificity and regulation of individual complexes (Cairns *et al.*, 1996b; Wang *et al.*, 1996). Yeast contains the SWI-SNF, ISW1 and ISW2, and RSC complexes; *Drosophila* NuRF, CHRAC and ACF, while the BRG1 and hbrm complexes have been found in mammalian cells (Burns and Peterson, 1997b; Cairns *et al.*, 1996a, 1996b, 1998; Kadonaga, 1998; Tsukiyama *et al.*, 1999). The conserved nature of these ATP-dependent remodeling complexes underlines their importance in eukaryotic Pol II transcription.

SWI-SNF and RSC can remodel nucleosomes with trypsinized core histones; thus, interactions of either of these complexes with the tail domains of core histones are not essential for remodeling (Guyon *et al.*, 1999; Logie *et al.*, 1999).

SWI-SNF. The SWI-SNF multisubunit complex consists of at least 11 proteins, of which four, Snf2p/Swi2p, Snf5p, Swi3p, and Swp73p, are conserved in other eukaryotic SWI-SNF-related complexes (Cairns *et al.*, 1994; Peterson *et al.*, 1994; Workman and Kingston, 1998). It is a non-essential complex that binds chromatin in a reaction involving the tails of the core histones (Georgel *et al.*, 1997; Wolffe and Guschin, 2000) and Sin1p (Moreira and Holmberg, 2000; Pérez-Martín and Johnson, 1998). Histone-DNA and histone-histone interactions are consequently destabilized to facilitate transcription factor binding, thereby making the chromatin template permissive for transcriptional regulation (Burns and Peterson, 1997a; Côté *et al.*, 1994, 1998; Imbalzano *et al.*, 1994; Logie and Peterson, 1997). To this end, positioned nucleosomes cover essential DNA-binding sites in the yeast *HO* promoter. SWI-SNF and SAGA are required to bind the activator Swi4p/Swi6p to these nucleosomal sites (Cosma *et al.*, 1999). Also, SWI-SNF targets promoter-proximal nucleosomes for disruption to facilitate transcriptional initiation by overcoming the inhibitory effect of nucleosomes on PIC formation (Hassan *et al.*, 2001).

SWI-SNF is not very abundant (~100-200 complexes/haploid cell) when compared to Pol II holoenzyme complexes (~3000 complexes/haploid cell) (Cairns *et al.*, 1996b; Wilson *et al.*, 1996). SWI-SNF is also partially redundant with other nucleosome modifying proteins, such as Gcn5p (Holstege *et al.*, 1998; Sudarsanam *et al.*, 2000).

Swi2p/Snf2p is a DNA-dependent ATPase essential for SWI-SNF function (Laurent *et al.*, 1993). Yeast strains devoid of Swi2p show both positive and negative changes to gene expression, indicating that the SWI-SNF complex is involved in both transcriptional activation and repression (Holstege *et al.*, 1998; Sudarsanam *et al.*, 2000).

Several interesting characteristics of SWI-SNF have recently been identified (Sudarsanam *et al.*, 2000). Firstly, the transcriptional dependence of promoters on SWI-SNF differs in different growth conditions, indicating a role for SWI-SNF in nutrient-mediated transcriptional regulation. Secondly, no motifs common to all the SWI-SNF dependent promoters exist, suggesting that no single DNA motif exerts SWI-SNF dependence on a promoter. Lastly, transcriptional control by SWI-SNF is at the level of individual genes, and not at the level of chromosomal domains.

RSC complex. RSC is a 15-subunit complex that also remodels chromatin in an ATP-dependent manner. It is similar to SWI-SNF as at least three RSC subunits, Sth1p, Rsc6p and Rsc8p, are similar to Swi2p/Snf2p, Swp73p, and Swi3p, respectively. Despite these similarities, significant differences include that: i) RSC is an essential multisubunit complex that is at least 10-fold more abundant than SWI-SNF (Sengupta *et al.*, 2001), ii) unlike SWI-SNF, it does not associate with the Pol II holoenzyme (Cairns *et al.*, 1996b; Wilson *et al.*, 1996), iii) inducible genes (*PHO8* and *SUC2*) that are transcriptionally impaired by *swi/snf* mutations are not affected by *rsc* mutations (Du *et al.*, 1998; Moreira and Holmberg, 1999), and iv) RSC has a repressing effect on the transcription of *CHA1* in yeast. RSC possibly increases the function or accessibility of a corepressor to the *CHA1* promoter (Moreira and Holmberg, 1999). In addition, the human analogue of the Swi2p, hbrm, acts as a specific corepressor of E2F1 (Trouche *et al.*, 1997).

The Swi2p/Snf2p of SWI-SNF and the Rsc4p of RSC interact with DNA in a nucleosome-dependent manner and are displaced from the DNA after remodeling. However, Sth1p, the RSC ATPase, also directly interacts with nucleosomal DNA, but it is not displaced after remodeling (Sengupta *et al.*, 2001). These differences indicate different mechanisms of action and different functions of the two chromatin remodeling complexes.

Recruiting remodeling complexes. Remodeling complexes can be recruited to specific promoter regions via different methods. The SWI-SNF complex can be recruited by specific DNA-bound activators or repressors (Cosma *et al.*, 1999; Dimova *et al.*, 1999; Natarajan *et al.*, 1999; Neely *et al.*, 1999; Yudkovsky *et al.*, 1999), the general transcription machinery (Cho *et al.*, 1998; Wilson *et al.*, 1996), and can also bind DNA structure (Bazett-Jones *et al.*, 1999; Côté *et al.*, 1994, 1998; Quinn *et al.*, 1996).

SWI-SNF is recruited to the promoter by the transcriptional activator Swi5p (Fig. 5). SWI-SNF remodels the nucleosomes to facilitate, in combination with Swi5p, the binding of SAGA followed by nucleosome hyperacetylation. In combination with SWI-SNF, SAGA recruits the transcriptional activator Swi4p/Swi6p to the promoter to activate transcription. This represents an ordered recruitment of transcription factors and chromatin remodeling complexes to a single promoter to ultimately combine in the transcriptional regulation of the target gene (Cosma *et al.*, 1999). Holstege *et al.* (1998)

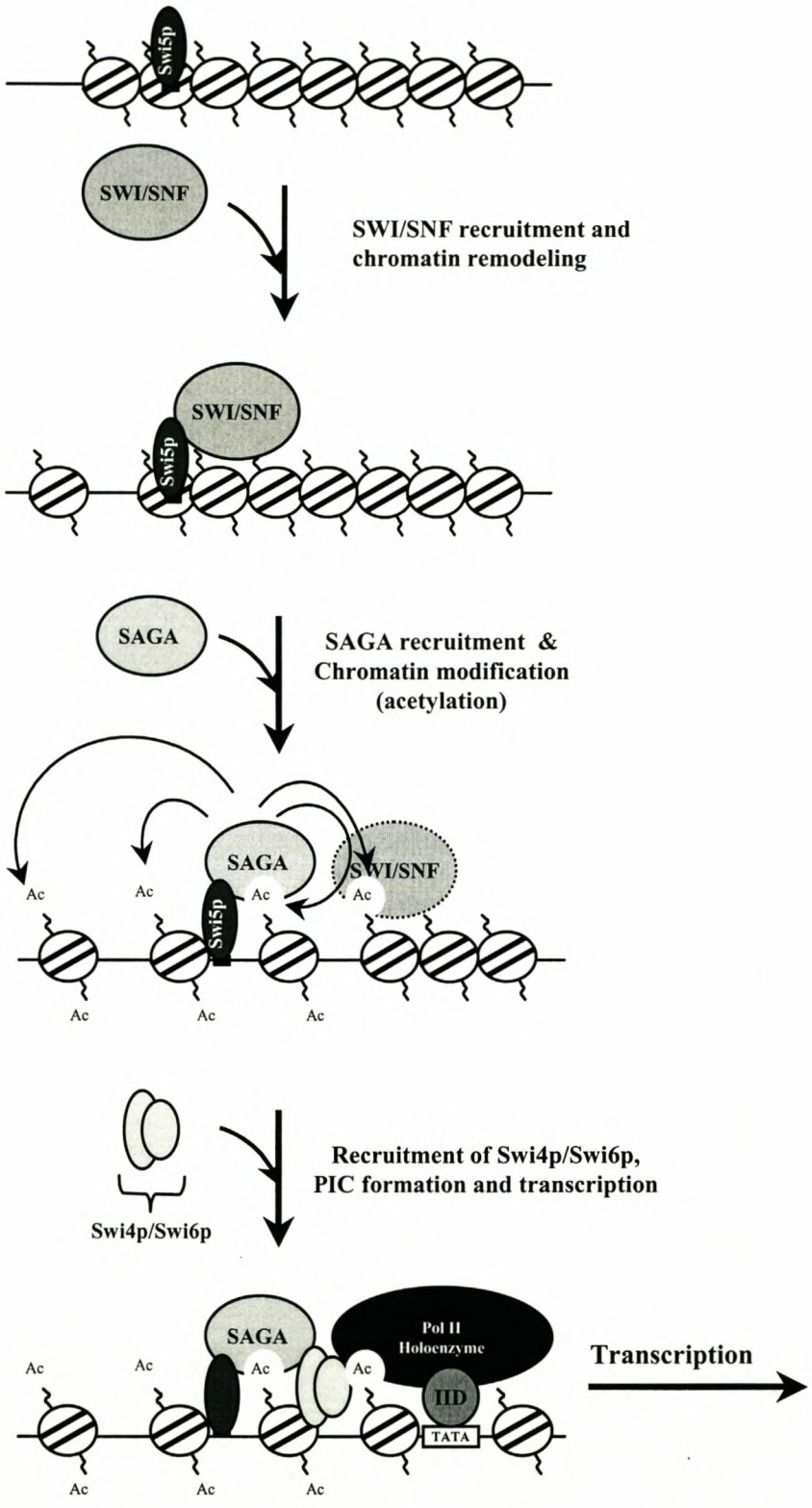

Fig. 5. The ordered recruitment of chromatin altering complexes required for the transcriptional activation of the yeast *HO* promoter (adapted from Fry and Peterson, 2001).

used genome-wide expression analysis to show that many genes dependent on SWI-SNF also require SAGA for transcription, thereby demonstrating a functional link between covalent and non-covalent chromatin remodeling and transcriptional regulation.

The SAGA-mediated acetylation of the *HO* promoter nucleosomes stabilizes the association of SWI-SNF with the promoter (Cosma *et al.*, 1999). The requirement of acetylated histones to stabilize the association of SWI-SNF with the promoter questioned the order in which the ATP-dependent remodeling and acetylation complexes are recruited to the promoter. The subsequently observations that Gcn5p recruitment and histone acetylation occur prior to SWI-SNF recruitment to higher eukaryotic promoters, confirmed that the recruitment of these complexes occurs in both ways (Agalioti *et al.*, 2000; Dilworth *et al.*, 2000; Hassan *et al.*, 2001).

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CHAPTER 2

Nitrogen catabolism in *Saccharomyces cerevisiae*

CONTENTS

INTRODUCTION

Living organisms require organic nitrogen to synthesize macromolecules, such as amino acids, proteins and nucleic acids, required for cellular functioning. *S. cerevisiae* can use a wide variety of nitrogenous compounds as a sole source of organic nitrogen. Ultimately, these compounds are degraded to ammonia or glutamate that, along with glutamine, serve as the interface between nitrogen catabolism and anabolism (Cooper, 1982a; Magasanik, 1992).

Among the multiple targets of metabolic regulation, *S. cerevisiae* uses transcriptional regulation as one of the primary mechanisms to adapt its metabolism to its environment. *S. cerevisiae* has the ability to distinguish between rich and poor nitrogen sources and to adapt its metabolism to the quality of the nitrogen source available (Cooper, 1982a; Magasanik, 1992). Proteins in the target of rapamycin (TOR) signal transduction pathway transduce the stimulus of the available nitrogen to various transcription factors (Beck and Hall, 1999; Cardenas *et al.*, 1999; Hardwick *et al.*, 1999; Shamji *et al.*, 2000). The activities of these proteins are regulated to control the expression of target genes in correlation with the environmental conditions. Nitrogen metabolism in *S. cerevisiae* and the control of nitrogen-regulated gene expression will be reviewed in this chapter.

NITROGEN METABOLISM

Nitrogen metabolism in *S. cerevisiae* entails the use of the available nitrogen source(s) to synthesize nitrogenous macromolecules needed for general cellular functions (Fig. 6). Intermediates of the ammonia-glutamate-glutamine interconversion pathway are major precursors or nitrogen donors in many anabolic reactions (Cooper, 1982a; Magasanik, 1992). Should any of these precursors become depleted, alternative nitrogen sources are catabolized to yield intermediates of the ammonia-glutamate-glutamine interconversion pathway. A variety of nitrogenous compounds can serve as a sole source of organic nitrogen. These include ammonia, a variety of amino acids, polyamines, ornithine, γ-aminobutyric acid, allantoin and urea.

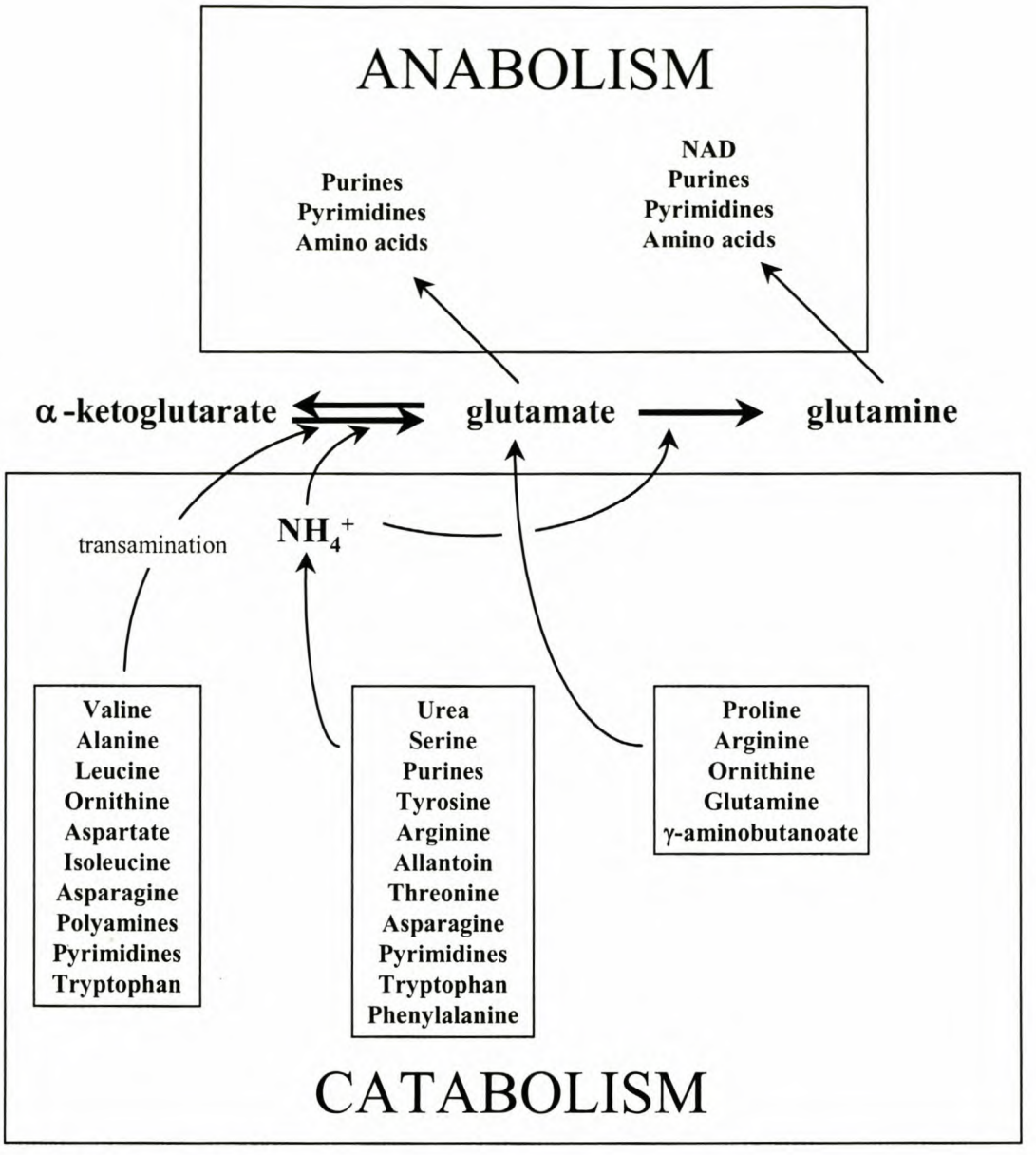


Fig. 6. Nitrogen metabolism in *S. cerevisiae.*

Nitrogen Catabolism

S. cerevisiae continuously needs to replenish precursors for the biosynthesis of essential macromolecules. Some nitrogen sources are metabolized much more efficiently than others. Thus, the rate at which nitrogenous compounds support the synthesis of macromolecules and consequently growth, determines the effectiveness of a nitrogen compound as a sole nitrogen source. When used as sole nitrogen sources, it is clear that some compounds support faster growth rates than others. Rich or preferred nitrogen sources (e.g. ammonia and glutamine) support a faster growth rate and more efficient metabolism than poor or alternative nitrogen sources (e.g. proline, allantoin

and threonine) (Cooper, 1982a; Magasanik, 1992). Preferred nitrogen sources enter the cell rapidly, are assimilated rapidly and do not have any toxic side effects on the cell. The classification of a nitrogen source as either rich or poor is dependent on the specific genetic composition and growth conditions of the yeast. Any characteristic that impedes with the metabolism of a specific nitrogen source would decrease its effectiveness (Cooper, 1982a).

The catabolism of various nitrogenous compounds has been studied extensively. Some catabolic pathways (proline and ornithine catabolism) lead to the production of glutamate, others (allantoin and urea degradation) lead to the production of ammonia while arginine catabolism replenishes both ammonia and glutamate (Fig. 6) (Cooper, 1982a; Magasanik, 1992). The focus of this section will be on the allantoin catabolic pathway and the ammonia-glutamate-glutamine interconversion pathway.

Allantoin catabolism

Allantoin is a product of purine degradation by many organisms and can serve as a sole nitrogen source for *S. cerevisiae* (Cooper, 1982a). It can be retained in the vacuole up to a concentration of 1 mM and is a good storage compound as it has a high carbon:nitrogen ratio. Allantoin is only degraded when the external nitrogen sources are poor or limiting. Thus, degradation will not occur if a preferred nitrogen source is present in abundance in the environment.

Allantoin degradation in *S. cerevisiae* requires the protein products of the *DAL* and *DUR* genes. These genes are contained in three gene clusters spread over the organism's genome. The largest of these clusters is located on the right arm of chromosome IX. This cluster contains *DAL81, DAL1*, *DAL4*, *DAL2*, *DAL7* and *DAL3* (Cooper, 1982a) (Fig. 7A).

Allantoin degradative pathway. Five enzymatic reactions by four enzymes degrade allantoin to ammonia and CO_2 (Fig. 7B). A sixth reaction disposes of the reactive and toxic glyoxylate produced during allantoin degradation.

Allantoin is transported into the cell by the allantoin permease encoded by *DAL4* (Fig. 7B). Once inside the cell, allantoin is hydrolyzed by allantoinase, a hydrolase encoded by *DAL1*, to yield allantoate. Extracellular allantoate can also be transported into the cell via a specific allantoate permease encoded by *DAL5*. Allantoate is subsequently

hydrolyzed by the second hydrolase, allantoicase, encoded by *DAL2*, to yield urea and ureidoglycolate. The third hydrolase, ureidoglycolate lyase, is encoded by *DAL3* and converts ureidoglycolate to urea and

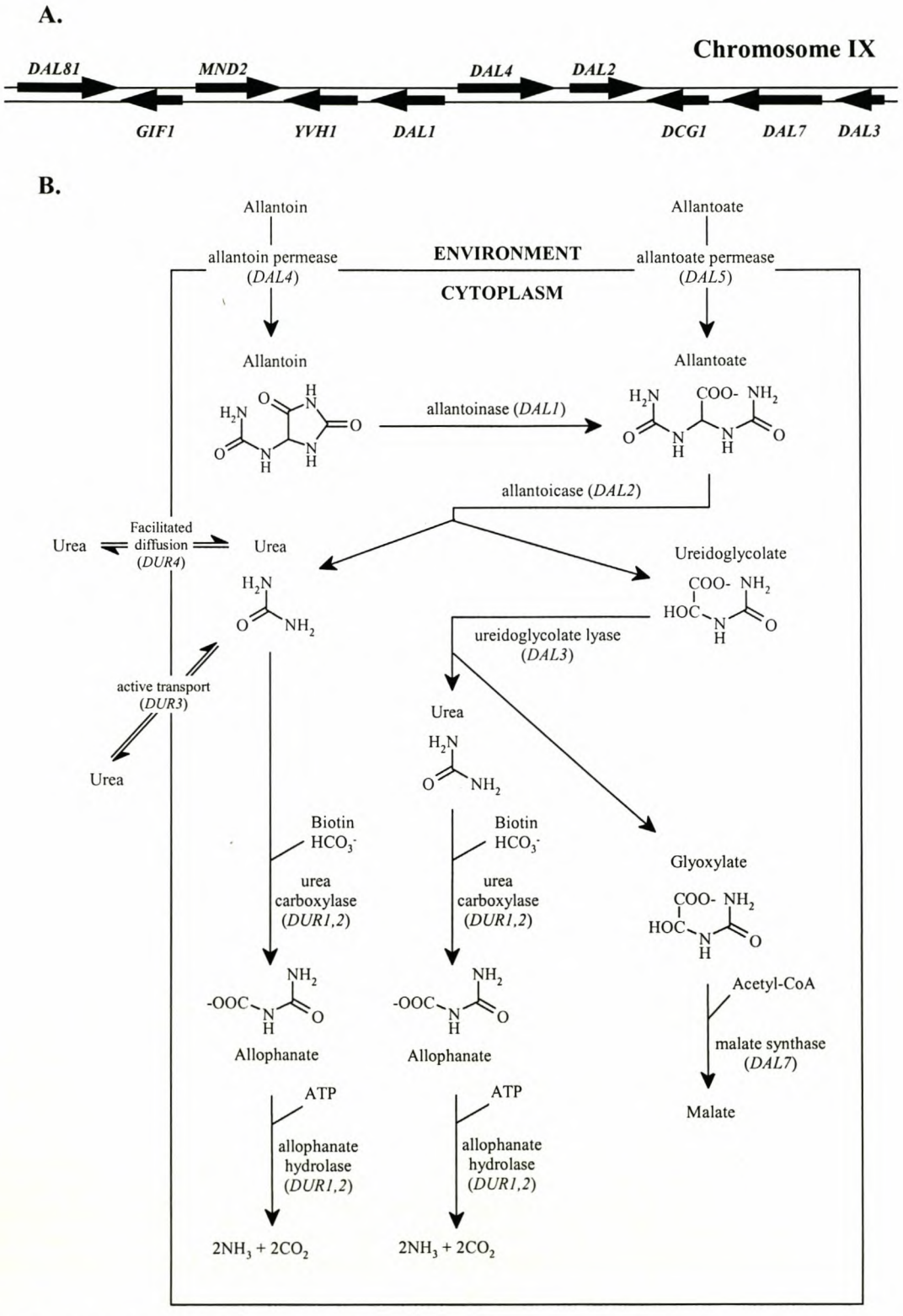


Fig. 7. Allantoin catabolism in *S. cerevisiae*. A) The *DAL* gene cluster on Chromosome IX, and B) the allantoin degradative pathway (adapted from Cooper, 1982a).

glyoxylate. Glyoxylate and acetyl-CoA are converted to malate by the malate synthase enzyme encoded by *DAL7* (Hartig *et al.*, 1992). This reaction ensures that glyoxylate, a reactive compound that is toxic to the cell, does not accumulate intracellularly (Cooper 1982a).

Both the allantoate and ureidoglycolate hydrolysis reactions yield one urea molecule. Thus, two molecules of urea are produced from one allantoin molecule. Environmental urea can also by transported into the cell by either of two transport systems. *DUR3* encodes a urea permease that actively transports urea when it is present in the environment at low concentrations (< 0.5 mM). When urea is present at high concentrations (>2.5 mM), the *DUR4* gene product enables its facilitated diffusion (Cooper, 1982b) (Fig. 7B).

While the urease enzyme of other organisms performs the hydrolysis of urea to produce ammonia, the urea carboxylase-allophanate hydrolase, a bifunctional enzyme, (encoded by *DUR1,2*) performs this function in *S. cerevisiae*. Urea is carboxylated by the ATP and biotin-dependent urea-carboxylase to yield allophanate. Allophanate becomes the substrate for the allophanate hydrolase to yield two molecules of ammonia and two molecules of CO_2. The two molecules of urea yielded via allantoin degradation therefore results in the production of four ammonia molecules that the yeast can use to synthesize nitrogen-containing compounds (Cooper, 1982a) (Fig. 7B).

Induction. The allantoin degradative pathway is only active in conditions of limited nitrogen supply (discussed later). In addition, it is induced by the last intermediate of the pathway, allophanate (Cooper, 1982a; Cooper and Lawther, 1973). Allophanate, or its analogue oxalurate (OXLU), enables induced expression of most *DAL* and *DUR* genes above its basal level of expression. The basal level of expression occurs in poor nitrogen conditions and in the absence of the inducer. However, allophanate is only produced after four enzymatic reactions of the allantoin degradative pathway. In addition, the allophanate hydrolase activity degrades allophanate five times faster than it is produced. These observations showed that a significant concentration of the precursor to allophanate synthesis, urea, had to be present in the cell to allow the synthesis of allophanate above the threshold needed for induction of the allantoin degradative pathway. In contrast to the inducible allantoin degrading enzymes, allantoin transport is induced not only by allophanate, but also by allantoin (Sumrada *et al.*, 1978). This dual induction of allantoin transport allows the cell to accumulate

sufficient allantoin intracellularly to produce sufficient allophanate to induce the allophanate-inducible genes. Thus, during limiting nitrogen conditions, environmental allantoin is accumulated in high concentrations in the cell. Allantoin degrading enzymes are present at relatively high basal concentrations in the cell, resulting in the increased conversion of allantoin to allophanate. Urea is a product of arginine catabolism and can also be actively transported into the cell by Dur3p (Cooper, 1982a).

Ammonia-Glutamate-Glutamine Interconversion Pathway

The ammonia-glutamate-glutamine interconversion pathway functions at the interface between nitrogen catabolism and anabolism. It allows the assimilation of ammonia and the synthesis of two essential amino acids in nitrogen metabolism, glutamate and glutamine (Fig. 8). Ter Schure *et al.* (2000) refers to the interconversion of ammonia, glutamate and glutamine as the Central Nitrogen Metabolism (ammonia-glutamate-glutamine interconversion pathway) as these reactions represent the interface of nitrogen catabolism and anabolism with the majority of nitrogen sources used. Each of the nitrogen intermediates of the interconversion pathway can serve as a sole nitrogen source for *S. cerevisiae*.

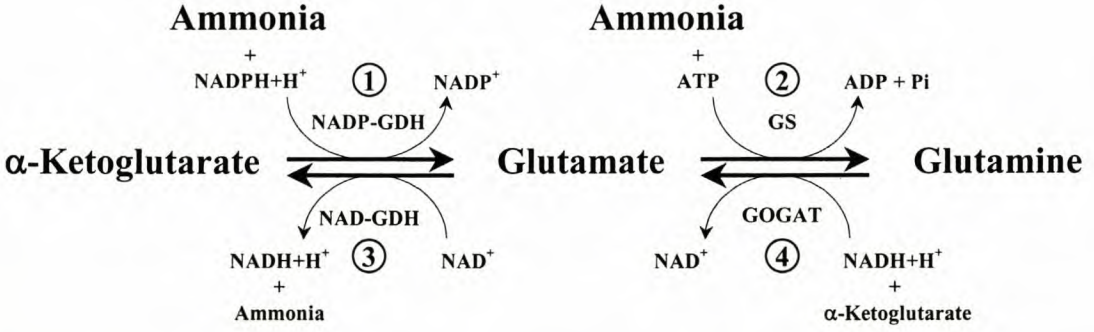


Fig. 8. The ammonia-glutamate-glutamine interconversion pathway in *S. cerevisiae* (adapted from Magasanik, 1992).

Ammonia

All nitrogen-containing molecules of the cell can be synthesized from ammonia and carbon intermediates obtained from the degradation of a major carbon source. Ammonia is transported into the cell via three known ammonium permeases encoded by *MEP1*, *MEP2*, and *MEP3*, respectively (Marini *et al.*, 1997). Intracellular ammonia

reacts with α-ketoglutarate, an intermediate of the TCA cycle, and NADPH to yield glutamate (Fig. 8 reaction 1) or with glutamate and ATP to form glutamine (Fig.8 reaction 2). These reactions are catalyzed by the NADP-dependent glutamate dehydrogenases (NADP-GDHs) encoded by *GDH1* and *GDH3*, and glutamine synthetase (GS) encoded by *GLN1*, respectively. The synthesized glutamate readily reacts with ammonia in the GS reaction, resulting in a glutamate deficiency of ammonia-grown cells. These observations explain why the growth rate of ammonia-grown cells is slower than that of glutamine-grown cells (Magasanik, 1992).

Glutamine

Glutamine supports the most rapid growth of yeast, as it is an excellent source of both glutamate and glutamine. Three broad-spectrum amino acid permeases, Gap1p, Dip5p, and Agp1p, and one specific amino acid permease, Gnp1p, mediate the transport of glutamine into the cell (Regenberg *et al.*, 1999). Glutamine is subsequently converted to glutamate by the action of *GLT1*-encoded glutamate synthase [GOGAT] (Fig. 8 reaction 4). A portion of the glutamate synthesized from glutamine arises from the use of the amide group of glutamine during the synthesis of nucleotides and certain amino acids. However, glutamate produced in this manner accounts for approximately 20% of the glutamate needed by the cell to support normal growth (Magasanik, 1992).

Glutamate

Glutamate is an essential amino acid in nitrogen metabolism by *S. cerevisiae*. Its transport is mediated by the two broad-spectrum amino acid permeases, Gap1p and Dip5p (Darte and Grenson, 1975; Regenberg *et al.*, 1998; 1999). However, the yeast grows slower with glutamate as sole nitrogen source compared to ammonia and glutamine, even though the amino nitrogen of glutamate supplies 88% of the cellular nitrogen. The remaining 12% of the nitrogen needed is supplied by ammonia for the synthesis of glutamine from glutamate by GS (Magasanik, 1992). When glutamate is the only nitrogen source, the ammonia needed to synthesize glutamine will be provided by cleavage of glutamate, which yields ammonia and α-ketoglutarate (Fig. 8 reaction 3). This reaction is catalyzed by a NAD-dependent glutamate dehydrogenase (NAD-GDH) encoded by *GDH2*. The only physiological role of NAD-GDH is to generate ammonia for the synthesis of glutamine. Glutamate-grown cells are consequently deficient in

glutamine production, thereby explaining the slower growth rate (Miller and Magasanik, 1990).

Glutamate can also be deaminated by Gad1p to yield α-aminobutyric acid, which is sequentially degraded by Uga1p and Uga2p to yield succinate and ammonia. In addition to its ability to donate nitrogen to various biosynthetic reactions, glutamate can therefore also supply intermediates for the TCA cycle (Coleman *et al.*, 2001; Kuruvilla *et al.*, 2001).

Biosynthesis of nitrogenous macromolecules

The synthesis of various amino acids and nucleotides is dependent on components of the ammonia-glutamate-glutamine interconversion pathway (Fig. 6). Glutamate and various α-keto acids serve as the substrates of transaminases in reactions that yield amino acids and α-ketoglutarate. One of these amino acids is aspartate, which is also an important precursor for the synthesis of a variety of other amino acids. Aspartate and glutamine are important precursors for the synthesis of nucleotides needed for nucleic acid production. It is therefore clear that the interconversion of ammonia, glutamate and glutamine and the regulation of these reactions are crucial for the cell to maintain active growth.

TRANSCRIPTIONAL REGULATION OF NITROGEN CATABOLISM

S. cerevisiae senses the composition of its environment and responds by adapting its physiology to ensure survival in its habitat. Similarly, the nutrient availability is monitored and metabolic pathways are regulated by either one or a combination of many control mechanisms to ensure that the yeast benefits maximally from the available nutrients. These control mechanisms include: (i) the transcriptional regulation of genes encoding various metabolism-related proteins, (ii) the translational regulation of mRNAs, (iii) the posttranslational regulation of the activities of various proteins, and (iv) the degradation of proteins in response to environmental conditions. All these processes have been extensively studied and also apply to nitrogen catabolism in *S. cerevisiae* (Beck and Hall, 1999; Berset *et al.*, 1998; Cardenas *et al.*, 1999; Cooper,

1982a; 1994; Cunningham *et al.*, 2000a; Grenson, 1983; Hardwick *et al.*, 1999; Magasanik, 1992; Schmidt *et al.*, 1998; Springael and André, 1998).

Two distinct pathways control the transcription of nitrogen-regulated genes in *S. cerevisiae*. The first pathway enables the yeast to detect and respond to different qualities of nitrogen sources (Cooper, 1982a; Hardwick *et al.*, 1999; Magasanik, 1992), while the second allows it to respond to the total absence of nitrogen (Hardwick *et al.*, 1999; Park *et al.*, 1996). The focus of this review will be on the first pathway.

Nitrogen-Sensitive Gene Expression

S. cerevisiae scavenges its environment for sources of organic nitrogen and has the ability to selectively degrade rich nitrogen sources before poor ones. The quality of the organic nitrogen source(s) available to the yeast can change dramatically. The yeast adapts to these changes primarily by regulating the transcription of genes involved in nitrogen metabolism. When an excess of rich nitrogen is available, the transcription of genes involved in the catabolism of poor nitrogen sources is repressed. Should only a poor nitrogen source be available, the transcription of these genes is generally derepressed. This phenomenon, which enables yeast to use nitrogenous compounds selectively, is termed Nitrogen Catabolite Repression (NCR) (Cooper, 1982a; Wiame *et al.*, 1985), nitrogen regulation (Magasanik, 1992) or nitrogen discrimination (Hardwick *et al.*, 1999).

NCR is a complex mechanism that involves a variety of diverse cytoplasmic proteins whose combined function controls the expression patterns of NCR-sensitive genes in correlation with the nitrogen supply. These proteins form a central signal transduction pathway that ultimately controls the functions of various transcription factors. The TOR signal transduction pathway senses and transduces the signal of available nitrogen to ultimately regulate the function of various transcriptional activators (Beck and Hall, 1999; Bertram *et al.*, 2000; Cardenas *et al.*, 1999; Hardwick *et al.*, 1999; Komeili *et al.*, 2000; Shamji *et al.*, 2000). Two of these activators, the GATA-type transcription factors Gln3p and Gat1p, are the major activators needed for the derepression of NCR-sensitive genes (Coffman *et al.*, 1996; Daugherty *et al.*, 1993; Stanbrough and Magasanik, 1996).

Transcriptional activators, like Gln3p and Gat1p, function in the nucleus by having their effects at various target promoters. The function of these activators can be regulated in various ways. Mechanisms that regulate the abundance of the activator in the nucleus include either controlling its abundance in the cell, or controlling its presence in the nucleus, or both. The TOR signal transduction pathway provides a mechanism to regulate the nuclear translocation of Gln3p and Gat1p in correlation with the nitrogen supply of the yeast (Beck and Hall, 1999; Cardenas *et al.*, 1999; Hardwick *et al.*, 1999; Shamji *et al.*, 2000). Also, once nuclear, the function of the activators can be regulated at its target promoters. Repressor proteins can compete with activators for binding to a shared DNA-binding site or obstruct the interaction of the activator with the basal transcription machinery to ultimately decrease the ability of the activator to function. A detailed review of the GATA factors and their action in the nucleus will follow later in this chapter.

In addition to NCR, some nitrogen sources and catabolic pathway intermediates can act as transcriptional inducers of specific pathway genes. While the transcription of all the nitrogen catabolic genes respond to the quality and abundance of the nitrogen source, only certain genes in respective nitrogen catabolic pathways will respond to the pathway-specific inducer.

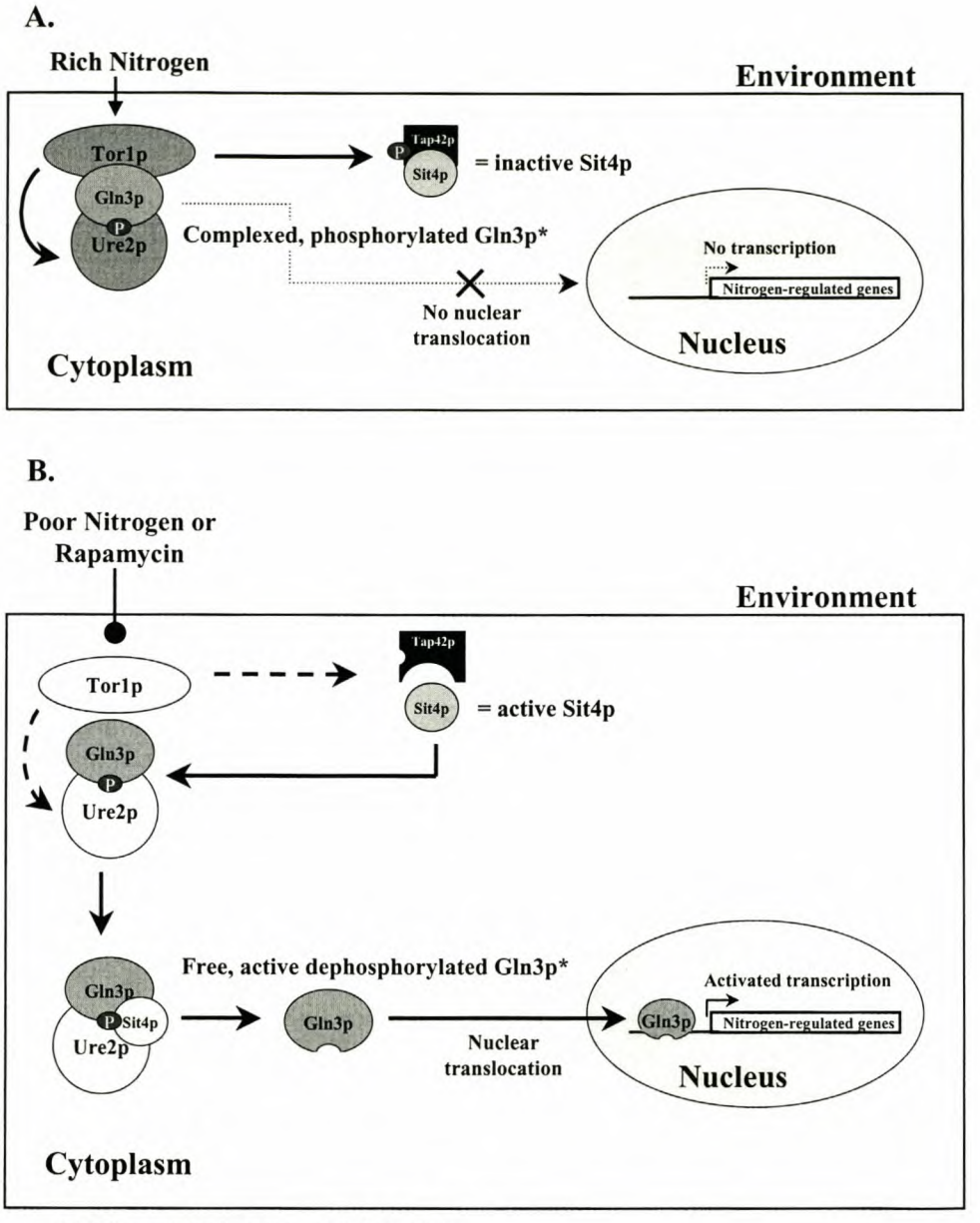
TOR signal transduction pathway

Yeast cells employ signaling pathways to sense and respond to their environment. One such pathway, the TOR signaling pathway, has been identified as the key mechanism in sensing and responding to environmental nutrient availability. Components of this pathway include a series of kinases, phosphatases and regulatory proteins that ultimately regulate the activity of Gln3p and Gat1p in response to the signal of nitrogen supply. The pathway is named after the two phosphatidylinositol kinase (PIK)-related kinases, Tor1p and Tor2p (TOR), which are central to its functions and highly conserved in eukaryotes (Kuruvilla and Schreiber, 1999). These kinases are essential for viability, regulated translation initiation and cell cycle progression (Barbet *et al.*, 1996; Berset *et al.*, 1998; Heitman *et al.*, 1991; Kunz *et al.*, 1993). In addition, they control: i) sporulation and autophagy by yeast cells in response to environmental nutrient conditions (Noda and Ohsumi, 1998; Zheng and Schreiber, 1997), ii) the function of the Npr1p, a protein kinase that controls the stability or degradation of

amino acid permeases (Schmidt *et al.*, 1998), and iii) the pathways required for carbon and nitrogen regulated transcriptional responses (Beck and Hall, 1999; Cardenas *et al.*, 1999; Hardwick *et al.*, 1999; Shamji *et al.*, 2000).

Rapamycin is a known macrolide antibiotic that specifically inhibits TOR (Cardenas *et al.*, 1999; Cutler *et al.*, 1999; Kunz *et al.*, 1993). It binds Fpr1p, the *S. cerevisiae* peptidylprolyl isomerase (PPIase) homologue of human FKBP12, with high specificity. This complex specifically binds the TOR FPR1-rapamycin binding domain and forms the FPR1-rapamycin-TOR ternary complex in which TOR is inactive (Cafferkey *et al.*, 1993; Kunz *et al.*, 1993; Zheng *et al.*, 1995). Rapamycin-induced inhibition of TOR results in an array of responses, one of which is the transcriptional activation of NCR-sensitive genes in the presence of an abundant rich nitrogen source (Beck and Hall, 1999; Cardenas *et al.*, 1999; Hardwick *et al.*, 1999; Shamji *et al.*, 2000). These genes are primarily activated by the actions of Gln3p and Gat1p (Coffman *et al.*, 1996; Magasanik, 1992; Stanbrough *et al.*, 1995). The expression patterns of NCR-sensitive genes respond similarly when yeast cells grown in rich nitrogen are either rapamycin-treatment or shifted to a poor nitrogen source, thereby confirming the correlation between nitrogen signaling and the TOR signaling cascade (Beck and Hall, 1999; Shamji *et al.*, 2000).

Kinases and phosphatases. The phosphorylated state of Gln3p and Gat1p seem to dictate their ability to function. Both activators are phosphorylated and cytoplasmic in the presence of abundant rich nitrogen sources (Fig. 9A) (Beck and Hall, 1999). The TOR kinase domain is essential for Gln3p phosphorylation, repression of Gln3p nucleocytoplasmic translocation and repression of Gln3p-dependent transcriptional activation (Bertram *et al.*, 2000). Once the yeast experiences either rapamycin treatment or limiting nitrogen conditions, Gln3p and Gat1p are dephosphorylated and translocate to the nucleus with the resulting increase in transcription of their target genes. Phosphorylated Gln3p and Gat1p cannot translocate to the nucleus; thus, dephosphorylation activates these GATA factors (Fig. 9B) (Beck and Hall, 1999; Shamji *et al.*, 2000).



* Gat1p and Gln3p are regulated similarly

Fig. 9. Cytoplasmic regulation of nitrogen-regulated transcriptional activators in correlation with the quality of available nitrogen. A) Rich nitrogen inhibits the nuclear translocation of activators, while B) poor nitrogen conditions or rapamycin treatment enhances this process. Arrows indicate activation and balled ends represent inhibition.

At least two types of phosphatases, the Tap42-regulated phosphatases, such as Sit4p, and the general protein phosphatase Pph3p, are required for the TOR-controlled dephosphorylation of Gln3p and Gat1p. Tap42p is an important, but not exclusive, regulator of TOR-mediated gene expression (Beck and Hall, 1999; Bertram *et al.*, 2000; Di Como and Arndt, 1996; Jiang and Broach, 1999; Shamji *et al.*, 2000). It is a highly conserved, essential phosphoprotein whose phosphorylation is regulated by Tor2p (Jiang and Broach, 1999). It functions as a regulatory subunit of various protein

phosphatase 2A (PP2A)- and PP2A-related phosphatases. These include the PP2A subunits Sit4p, Pph21p and Pph22p (Di Como and Arndt, 1996). Phosphorylated Tap42p binds and inactivates the phosphatase Sit4p (Fig. 9A). Unphosphorylated Tap42p is inactive as it cannot bind and inactivate Sit4p (Fig. 9B) (Di Como and Arndt, 1996; Jiang and Broach, 1999). Similarly, dephosphorylation of Tap42p when complexed with Sit4p releases the active phosphatase from the complex (Jiang and Broach, 1999). The *tap42-11* and *sit4* mutants did not show induced dephosphorylation of either Gat1p or Gln3p in response to either rapamycin treatment or limiting nitrogen conditions (Beck and Hall, 1999; Shamji *et al.*, 2000). In addition, the *pph3* mutant showed decreased *GAP1* expression in response to rapamycin treatment (Bertram *et al.*, 2000). Together, these observations indicate that the GATA factors remain phosphorylated and inactive in rich nitrogen sources due to the repressed activity of the phosphatases. However, no direct evidence could be found that showed the direct involvement of either Sit4p or Pph3p in the dephosphorylation of Gln3p and Gat1p.

The TOR proteins contain several HEAT repeats. These repeats form elongated coiled-coil motifs that allow protein-protein interactions. The HEAT repeats of Tor1p physically interact with Gln3p and Gat1p. Tor1p binds Gln3p in the absence of Ure2p, while Ure2p is unable to interact with Tor1p in the absence of Gln3p. Thus, Gln3p facilitates the interaction of Ure2p with Tor1p. Four additional nitrogen-related transcriptional regulators, Deh1p, Dal80p, Dal81p and Dal82p, interact with Tor1p and Tor2p in a yeast two-hybrid system. Collectively these observations indicate that NCR-sensitive gene expression is ultimately controlled by the activity of the TOR proteins in response to the environmental nitrogen (Bertram *et al.*, 2000).

In addition, the HEAT repeats in Tor2p localizes this protein to the plasma membrane (Kunz *et al.*, 2000). Tor1p is also present in the plasma membrane, but its HEAT repeats were not implicated in its localization. The localization of these proteins to the plasma membrane is consistent with their central role in nutrient signaling in eukaryotes.

Ure2p and Mks1p. In addition to the kinases and phosphatases mentioned, other known regulators also function in NCR-sensitive gene expression. These include Ure2p and Msk1p. Deletion of *URE2* results in the derepression of NCR-sensitive genes in repressive nitrogen conditions (Coffman *et al.*, 1994; Coschigano and Magasanik, 1991). It is a cytoplasmic protein that inhibits NCR-sensitive gene expression by

regulating the functions of Gln3p and Gat1p (Coffman *et al.*, 1994; Cunningham *et al.*, 2000a; Shamji *et al.*, 2000). In addition, Ure2p is required to establish ion tolerance (Withee *et al.*, 1998), pseudohyphal growth (Lorenz and Heitman, 1998), and has the ability to form prions ([URE3]) in *S. cerevisiae* (Masison and Wickner, 1995; Wickner, 1994). Prions are altered forms of normal cellular proteins. These alterations normally result in the loss of function of the normal protein while the prion is infectious, thereby converting normal forms of the protein into prions (Prusiner, 1994). [URE3] and *ure2* mutant strains both fail to regulate the expression of NCR-sensitive genes in repressive nitrogen conditions (Coffman *et al.*, 1994; Coschigano and Magasanik, 1991; Lacroute, 1971). However, the repressive effect of Ure2p on NCR-sensitive gene expression in rich nitrogen conditions is not regulated by prion formation (Maddelein and Wickner, 1999; Masison *et al.*, 1997).

Although Ure2p interacts with Gln3p and regulates its function (Beck and Hall, 1999; Blinder *et al.*, 1996; Coffman *et al.*, 1994), Beck and Hall (1999) reported no significant complex formation between Gat1p and Ure2p. However, Gat1p nucleocytoplasmic translocation and Gat1p-mediated transcription are regulated by Ure2p (Cunningham *et al.*, 2000a). Ure2p therefore governs the functions of both activators in correlation with the nitrogen supply.

It seems unlikely that the phosphorylated state of Gln3p would influence its interaction with Ure2p as the repressor binds both hyper- and dephosphorylated Gln3p (Bertram *et al.*, 2000). Also, dephosphorylation of Gln3p does not result in its automatic dissociation from its complex with Ure2p. However, Ure2p limits Gln3p dephosphorylation, as Ure2p-bound Gln3p is more resistant to dephosphorylation than free Gln3p.

Like Gln3p, Ure2p is also a phosphoprotein that is phosphorylated by Tor1p in rich nitrogen conditions. Also, rapamycin-treatment or nitrogen limitation results in the dephosphorylation of Ure2p. Thus, TOR regulates the phosphorylated state of Ure2p in correlation with the nitrogen supply (Cardenas *et al.*, 1999; Hardwick *et al.*, 1999). Both Gln3p and Ure2p are targets for dephosphorylation in these conditions (Beck and Hall, 1999; Cardenas *et al.*, 1999; Hardwick *et al.*, 1999; Shamji *et al.*, 2000). The Ure2p-Gln3p complex is therefore the target for TOR-mediated dephosphorylation. However, the exact mechanism of release of Gln3p from the complex is currently unclear. Ure2p is dephosphorylated in the *tap42-11* mutant in response to rapamycin

treatment, while Gln3p is not (Shamji *et al.*, 2000). This observation indicates that the phosphorylated state of Ure2p is TOR-regulated, but independent of Tap42p. The TOR proteins therefore regulate the Ure2p-Gln3p complex via two distinct mechanisms (Fig. 9).

The above model explains a signaling pathway in which the environmental signal is transduced via Ure2p to Gln3p and/or Gat1p to regulate NCR-sensitive gene expression. This is supported by DNA array analysis that shows a) deletion of *URE2* is sufficient to activate the transcription of all NCR-sensitive genes in a rich nitrogen environment and b) the subsequent deletion of *GLN3* (*ure2gln3* double mutant) affects only a subset of NCR-sensitive genes. These observations indicate that not only Ure2p-dependent but also Gln3p-dependent and -independent NCR-sensitive transcription exists (Shamji *et al.*, 2000). However, this contradicts previous data that showed the *ure2* mutant to have a transcriptional phenotype for NCR-sensitive genes that ranged from complete NCR-sensitivity to complete NCR-insensitivity. The latter observations therefore support a model in which both Ure2p-dependent and -independent NCR-sensitive gene expression exists (Coffman *et al.*, 1994; 1995; 1996).

Mks1p was recently identified as a cytoplasmic inhibitor of Ure2p function and therefore a positive regulator of NCR-sensitive gene expression in derepressive nitrogen conditions. Epistasis experiments showed that Mks1p functions upstream of Ure2p and via the Ure2p-Gln3p signaling pathway (Edskes *et al.*, 1999). Mks1p was first identified as a negative regulator of growth in the Ras-cAMP pathway (Matsuura and Anraku, 1993). Feller *et al.* (1997) showed that *MKS1* and *LYS80*, a negative regulator of lysine biosynthesis, were identical. Some TCA cycle enzymes display increased activities in the *lys80* mutant. This leads in elevated α–ketoglutarate and glutamate levels in the mutant. The authors attribute the elevated lysine levels in the *lys80/mks1* mutant to the increased α–ketoglutarate levels. The different levels of nitrogen components in the ammonia-glutamate-glutamine interconversion pathway might therefore be involved in Mks1p-mediated regulation of Ure2p function. However, no data currently exists to support this statement.

Bertram *et al.* (2000) hypothesized that Mks1p might bind dephosphorylated Ure2p and possibly aid in the release of Gln3p from its complex with Ure2p. However, both the wild type and *mks1* mutant strains show Ure2p dephosphorylation and induced NCR-sensitive gene expression in response to rapamycin-treatment. TOR can therefore

govern NCR-sensitive gene expression independent of Mks1p. Collectively these data identify at least two possible mechanisms of nitrogen-related control of Ure2p: one that involves Mks1p and another that does not. No direct evidence exists that shows the involvement of Mks1p in the dissociation of the Ure2p-Gln3p complex.

Rtg1/3p and the Hap2/3/4/5p complexes are important activators of the early TCA cycle genes (Liu and Butow, 1999). TOR regulates the activities of both complexes (Fig. 10) (Shamji *et al.*, 2000). The activity of Rtg1/3p is also regulated by different nitrogen conditions. As the TCA cycle provides the α–ketoglutarate needed to synthesize glutamate, the availability of glutamate is essential for regulating the gene expression patterns of TCA cycle and nitrogen metabolic genes (Komeili *et al.*, 2000; Liu and Butow, 1999). TOR signals to the Rtg1/3p complex via Mks1p in a Tap42p-dependent manner (Shamji *et al.*, 2000). Thus, Mks1p is involved in both nitrogen and carbon-regulated gene expression.

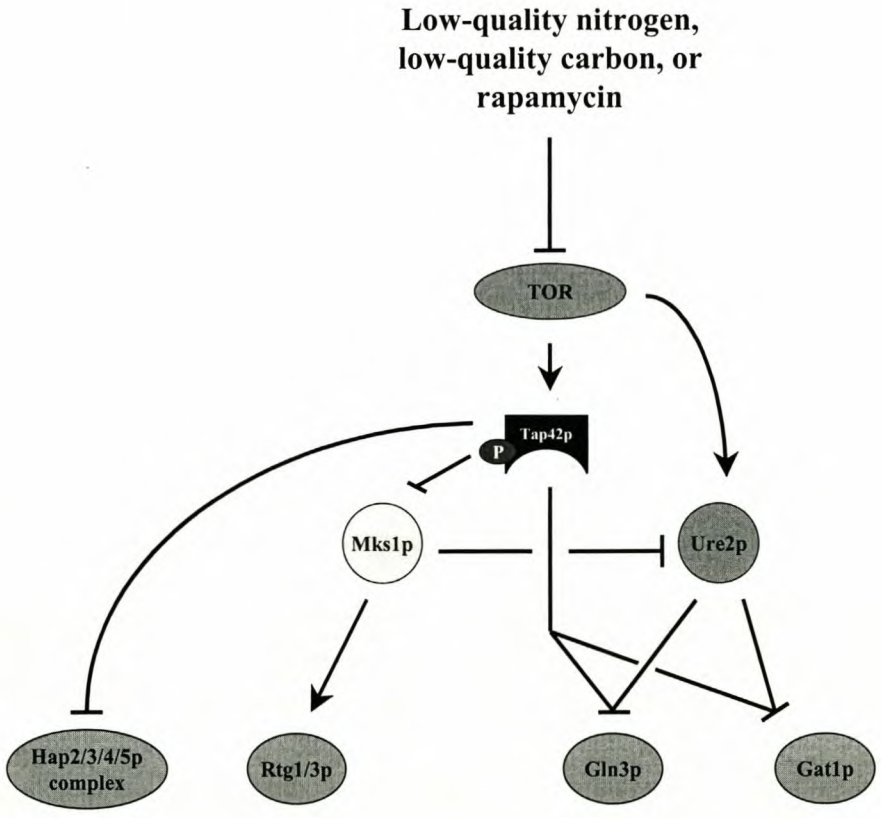


Fig. 10. TOR-mediated control of various nutrient-related transcriptional activators in *S. cerevisiae* (adapted from Shamji *et al.*, 2000).

Sensing nitrogen supply. The series of events in the TOR signaling cascade have been well studied. However, the initial event, sensing the quality of the nitrogen supply to activate or repress the signaling cascade, is still somewhat controversial. The range of nitrogen sources utilized by *S. cerevisiae* is degraded to either ammonia or glutamate (Cooper, 1982a; Magasanik, 1992). The components of the ammonia-glutamate-glutamine interconversion pathway are central in nitrogen metabolism and are therefore prime target molecules for possible sensors that would transduce the nitrogen signal to the TOR cascade. Two molecules, glutamine (Magasanik, 1992; Marzluf, 1997) and ammonia (ter Schure *et al.*, 1998), are proposed as the pivotal molecules in nitrogen signal transduction. Signaling by either of these molecules requires Ure2p to establish nitrogen-sensitive gene expression (Magasanik, 1992; ter Schure *et al.*, 1998).

Glutamine was originally identified as the central molecule that dictates the transcriptional profile of the yeast in response to its nitrogen supply. An abundantly rich nitrogen supply results in high intracellular concentrations of glutamine (Magasanik, 1992). Ure2p has been shown to bind and inhibit GS and Gln3p in rich nitrogen conditions (Blinder *et al.*, 1996; Coshigano and Magasanik, 1991; Courchesne and Magasanik, 1988; Xu *et al.*, 1995). When the yeast is shifted to poor or limited nitrogen conditions, intracellular glutamine decreases and Ure2p becomes inactive. GS and Gln3p are consequently active and nitrogen-regulated gene expression increases. This model therefore implies that intracellular ammonia has to be converted to glutamine to have its effect on nitrogen-regulated gene expression (Magasanik, 1992).

Mutation of *SUP70*, which encodes the glutamine $tRNA_{CUG}$, allows pseudohyphal growth, sporulation and derepressed expression of *CAR1* in rich nitrogen conditions, processes that would normally be repressed by these repressive nitrogen conditions (Murray *et al.*, 1998). The authors suggest that glutaminyl-$tRNA_{CUG}$ levels signal the nitrogen status in the cell. Thus, charged $tRNA_{CUG}$ corresponds to rich abundant nitrogen conditions and high concentrations of intracellular glutamine, while uncharged $tRNA_{CUG}$ depicts nitrogen limiting conditions and low intracellular glutamine concentrations. Beeser and Cooper (1999) confirmed the requirement for $tRNA_{CUG}$ in response to nitrogen starvation-related processes (pseudohyphal growth), but showed no need for $tRNA_{CUG}$ to establish NCR. The latter result confirmed the separation of nitrogen discrimination (NCR) from the nitrogen starvation response (pseudohyphal growth, sporulation, etc.) (Park *et al.*, 1996). To this end, Hardwick *et al.* (1999)

showed that inactivation of the TOR proteins by rapamycin induced the NCR-sensitive genes, but not nitrogen starvation-specific genes.

In contrast, ter Schure *et al.* (2000) state that the concentration of ammonia governs nitrogen-regulated gene expression independent of glutamine synthesis. The authors hypothesize two possible signals by which ammonia results in nitrogen regulation: the nitrogen flux in the ammonia-glutamate-glutamine interconversion pathway (i.e. ammonia to glutamate to glutamine) or the nitrogen concentration (i.e. ammonia concentration). NCR-sensitive genes are repressed in continuous culture conditions with ammonia (thus constant nitrogen flux) using a *gln1-37* mutant strain. Thus, NCR sensitivity is achieved without changes in the intracellular glutamine concentration. This repression was lost when the *gln1-3 ure2* double mutant was used. Collectively these observations show that the ammonia concentration represses nitrogen-regulated genes via Ure2p, but independent of glutamine synthesis. However, the authors have not been able to determine whether it is ammonia or glutamine concentrations that are sensed when glutamine is supplied as sole nitrogen source (ter Schure *et al.*, 1998).

The ammonium permease, Mep2p, is an ammonia-specific sensor required for pseudohyphal growth in response to ammonia starvation. The regulatory proteins Gln3p, Ure2p and Npr1p are also required for pseudohyphal growth in low proline and glutamine in addition to low ammonia conditions. Based on these observations Mep2p is proposed as a component of a nitrogen sensor that senses and signals suitable conditions for pseudohyphal growth (Lorenz and Heitman, 1998). Despite its clear role in pseudohyphal growth, Mep2p does not seem to function as a sensor for NCR. The activities of the ammonia-glutamate-glutamine interconversion pathway enzymes GS, NAD-GDH and NADP-GDH are similar in wild type and *mep2/mep2* mutant diploid strains (Lorenz and Heitman, 1998) and no data could be found that indicate the participation of Mep2p in the expression of NCR-sensitive genes.

Transcriptional regulators

Systematic analyses of the transcriptional regulation of various nitrogen catabolic pathway genes, including the *DAL* genes, have provided a useful model to study NCR-sensitive gene expression. All the *DAL* genes are NCR-sensitive and a common outline for their regulation has been identified. Detailed analyses of the promoter regions of these genes identified *cis*-acting elements that are at the center of NCR-sensitive

transcriptional regulation (Cooper, 1994). The GATA family of transcription factors was identified as regulators in NCR-sensitive gene expression via these elements (Coffman and Cooper, 1997; Coffman *et al.*, 1997; Cunningham and Cooper, 1991; Cunningham *et al.*, 1996; Soussi-Boudekou *et al.*, 1997; Stanbrough and Magasanik, 1996).

The GATA family of transcription factors consists of proteins that contain one or two tandem copies of the GATA zinc finger domain, Cys-X_2-Cys-X_{17}-Cys-X_2-Cys (Evans and Felsenfeld, 1989). These domains directly bind 5'-GATA-3' core DNA sequences (Omichinski *et al.*, 1993) and are highly conserved in eukaryotic organisms. In fungal organisms the majority of these GATA factors are global regulators of genes involved in nitrogen metabolism (Coffman *et al.*, 1996; Coornaert *et al.*, 1992; Cunningham and Cooper, 1991; Fu and Marzluf, 1990; Kudla *et al.*, 1990; Minehart and Magasanik, 1991; Stanbrough *et al.*, 1995). The four GATA factors associated with nitrogen metabolism in *S. cerevisiae*, Gln3p, Gat1p/Nil1p, Dal80p/Uga43p and Deh1p/Nil2p/Gzf3p, can be separated into two groups according to their effects on the transcriptional regulation of NCR-sensitive genes. Gln3p and Gat1p are two transcriptional activators required as mentioned previously (Coffman *et al.*, 1996; Cunningham *et al.*, 1994; 1996; Stanbrough and Magasanik, 1996). In contrast, the two transcriptional repressors, Dal80p and Deh1p, repress Gln3p- and Gat1p-dependent transcriptional activation, respectively. In addition, Dal80p also down-regulates Gat1p-dependent activation (André *et al.*, 1995; Coffman *et al.*, 1997; Daugherty *et al.*, 1993; Soussi-Boudekou *et al.*, 1997).

Gln3p and Gat1p/Nil1p. Gln3p, a strong transcriptional activator, was originally the main focus in studying NCR-sensitive derepression of nitrogen catabolic genes (Cooper *et al.*, 1990; Minehart and Magasanik, 1991). However, Gln3p-independent NCR-sensitive gene expression led to the identification of Gat1p, a potentially weaker transcriptional activator that functions in a manner similar to Gln3p (Coffman *et al.*, 1995; 1996).

Two lines of evidence show that these two activators function in a manner that maximally benefits the yeast's physiology in a specific nitrogen environment. Although Gln3p and Gat1p facilitate the maximal derepression of various nitrogen catabolic genes, the contribution of each activator to derepression is gene-specific. It can be i) highly dependent on both activators (i.e. *GAP1*) (Stanbrough and Magasanik, 1995;

Stanbrough *et al.*, 1995; 1996), ii) more Gln3p- and less Gat1p-dependent (i.e. *GLN1*), or iii) fully Gln3p-dependent, (i.e. *GDH2*) (Coffman *et al.*, 1996; Stanbrough *et al.*, 1995). In addition, the functions of both these activators are regulated by the supplied nitrogen source (Coffman *et al.*, 1995; 1996; Stanbrough and Magasanik, 1995). Gln3p is inactive in ammonia (when the intracellular glutamate and glutamine levels are low and high, respectively) and active in glutamate (when the intracellular glutamate and glutamine levels are high and low, respectively), while Gat1p is active in ammonia and inactive in glutamate. Both activators are inactive in glutamine-grown cells (abundant glutamate and glutamine). In contrast, both activators are active when proline and urea are used as nitrogen sources as the cells shift between glutamate and glutamine limited growth. Collectively these observations are consistent with the physiology of the cell in response to a specific nitrogen supply. For example, the NAD-GDH is required to generate ammonia from glutamate. Gln3p, which is active in glutamate, activates the expression of this gene in glutamate-grown cells. If *GDH2* expression were Gat1p-dependent, it would not have been transcribed with glutamate as nitrogen source as Gat1p is not active.

Gln3p and Gat1p share protein homologies. In addition to the highly conserved GATA zinc finger domain, both proteins have i) highly acidic amino terminal regions (residues 1-160) (Minehart and Magasanik, 1991; Stanbrough *et al.*, 1995), characteristic of transcriptional activators (Ptashne, 1988), and, ii) asparagine-rich regions between residues 60 and 308. Both these regions are absent from Dal80p and Deh1p (Stanbrough *et al.*, 1995).

Three observations argue that Gln3p might be involved in processes other than the transcriptional regulation of nitrogen-regulated genes. Firstly, *GNP1* (glutamine permease) expression is highly dependent on Gln3p, irrespective of the nitrogen source available to the cell and despite the absence of GATA elements from its promoter (Zhu *et al.*, 1996). Secondly, *gln3* strains have slow growth phenotypes in a variety of nutritional conditions (Smith *et al.*, 1996). Lastly, the increased resistance of the *ure2* mutant to high salt concentrations requires the presence of a functional Gln3p, thereby implying a role for this activator in establishing ion tolerance in *S. cerevisiae* (Withee *et al.*, 1998). Therefore, Gln3p does not seem to be totally inactive in rich nitrogen conditions and might not be limited to its effect on nitrogen-regulated genes.

cis-*Acting elements*. Several *cis*-acting elements are involved in the transcription of nitrogen-regulated genes. These include: i) elements needed to facilitate NCR-sensitive gene expression, ii) global transcription activator binding sites that activate the transcription of their target genes independent of nitrogen source, and iii) elements identified that function in NCR-sensitive gene expression, but do not yet have specific proteins associated with them.

The *cis*-acting element at the centre of NCR-sensitive transcriptional activation is the positively acting UAS_{NTR} (Upstream Activation Sequence NiTrogen-Regulated). It is a dodecanucleotide element with a 5'-GATAA-3' core sequence (Bysani *et al.*, 1991; Rai *et al.*, 1989). Two or more copies of this element are required for transcriptional activation and multiple copies are present in the promoter regions of NCR-sensitive genes (Blinder and Magasanik, 1995; Bysani *et al.*, 1991; Cunningham *et al.*, 1996; Daugherty *et al.*, 1993; Rai *et al.*, 1989). UAS_{NTR} elements are both necessary and sufficient for NCR-sensitive transcriptional activation of all the *DAL* genes (Cooper *et al.*, 1989; 1990). UAS_{NTR}-mediated activation requires either Gln3p or Gat1p or both activators (Coffman *et al.*, 1996; 1997; Cooper *et al.*, 1990; Soussi-Boudekou *et al.*, 1997; Stanbrough and Magasanik, 1996). The elimination of the GATAAG elements from the *GAP1* promoter affected the activation of transcription by both Gln3p and Gat1p. Thus, these activators recognize the same DNA binding sites (Stanbrough and Magasanik, 1996). Only Gln3p has been shown to bind to the UAS_{NTR} elements (Blinder and Magasanik, 1995; Cunningham *et al.*, 1996). However, the strong homology that exists between the zinc finger DNA-binding domains of the Gln3p and Gat1p, as well as the overlapping function of these activators at various UAS_{NTR} elements, strongly suggests that Gat1p also bind these elements (Coffman *et al.*, 1996; 1997; Cunningham *et al.*, 1996; Minehart and Magasanik, 1991; Stanbrough and Magasanik, 1996).

The *CAN1* and *DAL* genes contain multiple UAS_{NTR} elements in their promoter regions, but *CAN1* is much less NCR-sensitive than the *DAL* genes (Daugherty *et al.*, 1993). More than just the presence of the UAS_{NTR} elements is therefore needed for i) NCR-sensitive gene expression and ii) regulated derepression of NCR-sensitive genes.

A second element, 5'-TTG(T/G)T-3', was identified in the promoters of *GLN1*, *GDH2* and *GAP1*. This element plays an auxiliary role in the Gln3p-mediated activation of *GLN1* and *GDH2* (Miller and Magasanik, 1991; Minehart and Magasanik,

1991; Rai *et al.*, 1995). Analysis of the *GAP1* promoter showed that single copies of the two *cis*-acting elements, UAS_{NTR} and 5'-TTG(T/G)T-3', are needed for Gln3p-facilitated transcriptional activation. These two sites, as well as an Abf1p binding site (discussed below), are needed for a *GAP1* transcriptional response to Gat1p (Stanbrough and Magasanik, 1996). However, the 5'-TTG(T/G)T-3' element alone cannot activate transcription without the presence of the UAS_{NTR} (Rai *et al.*, 1995).

Two global regulatory factors (GRFs), Abf1p and Rap1p, activate the transcription of various unrelated genes. Rap1p also has roles in transcriptional silencing and telomere maintenance (Shore, 1994). These proteins often function together via UAS elements that are located in close proximity to each other (Walker *et al.*, 1990). These elements function independent of the nitrogen source supplied, thereby implying that these factors function by contributing to the basal strength of the promoter in which they are located.

Abf1p serves as a nucleosomal boundary element by organising nucleosomes into ordered arrays. The presence of a T-rich element [or poly(dA-dT) tracts] flanking the Abf1p binding site creates a nucleosome-free region (Lascaris *et al.*, 2000). It is required for i) the silencing of transcription of the silent mating type locus *HMR* (Borrelli *et al.*, 1984), ii) transcriptional activation of the ribosomal protein genes (Planta *et al.*, 1995), *COX6* (Silve *et al.*, 1992), *ARO3* (Kunzler *et al.*, 1995), *FOX3* (Einerhand *et al.*, 1991), *CHA1* (Bornaes *et al.*, 1993), *HIS7* (Springer *et al.*, 1997), *BAP3* (de Boer *et al.*, 2000), *ENO1* and *ENO2* (Brindle *et al.*, 1990), and iii) DNA replication (Marahrens and Stillman, 1992). In addition, an Abf1p binding site, with the consensus sequence of 5'-RTCRYYNNNNACG-3' (Brindle *et al.*, 1990), was found in the promoters of *GAP1*, *CAR1* and *CAR2* (Park *et al.*, 1999; Smart *et al.*, 1996; Stanbrough and Magasanik, 1996). This element enhances Gat1p-dependent, but not Gln3p-dependent, transcriptional activation of *GAP1* (Rowen *et al.*, 1997; Stanbrough and Magasanik, 1996).

The Rap1p binding site (consensus sequence 5'-RTRCACCCANNCMCC-3') is found in the promoter regions of various genes, and normally in combination with other transcription factor binding sites (Drazinic *et al.*, 1996; Shore, 1994). This element alone has very little capacity to activate the transcription of its target gene, but mutation thereof greatly reduces the promoter's ability to activate transcription (Drazinic *et al.*,

1996). Rap1p interferes with nucleosome positioning, thereby affecting the chromatin structure of the promoter regions of genes (Yu and Morse, 1999). It opens chromatin and exposes weak activator binding sites, consequently enabling the binding of various activators to their target sequences (Drazinic *et al.*, 1996; Yu and Morse, 1999). Rap1p *cis*-acting elements are present in the *CAR1* and *CAR2* promoter regions and needed for the maximal expression of these genes (Park *et al.*, 1999; Smart *et al.*, 1996).

A fifth element implicated in the transcriptional regulation of some NCR-sensitive genes is the GC-rich regions found in the promoter regions of the *CAR1* and *DAL7* genes (Rai *et al.*, 1999; Smart *et al.*, 1996). It has the sequence 5'-CCGCGG-3' and shares two characteristics commonly found in various transcription factor DNA-binding sites. It is GC-rich and has an inverted repeat. Mutation of this region in the *DAL7* promoter showed a decrease in both the derepressed and induced expression (discussed below), showing that it is needed for derepression of this gene (Rai *et al.*, 1999). This element also has a positive effect on the expression of *CAR1* (Smart *et al.*, 1996). The precise mechanism by which this element acts and which proteins mediate its function are currently not clear.

Co-activator. Ada1p is a component of the ADA/GCN5 co-activator complex (Horiuchi *et al.*, 1997) and is required for the structural integrity of the SAGA complex (Sterner *et al.*, 1999). The *ada1* mutant has a decreased ability to acetylate nucleosomes and have severe growth defects (Horiuchi *et al.*, 1997; Sterner *et al.*, 1999), including a slow growth phenotype on several nitrogen sources (Soussi-Boudekou and André, 1999).

The *ada1* mutant supports lower levels of derepression of many NCR-sensitive genes. These include *GLN1*, *GDH2*, *PUT4*, *GAP1* and *MEP2*. As the ADA/GCN5 and SAGA complexes can function by linking transcriptional activators with the basal transcription apparatus, Ada1p/Gan1p was studied in relation to Gat1p and Gln3p. The co-activator is essential for Gat1p-dependent transcription, while it is in limited demand for Gn3p-dependent transcription. These results show a need for Ada1p-containing complexes (ADA/GCN5 and/or SAGA) in NCR-sensitive gene expression (Soussi-Boudekou and André, 1999). The exact role of such a complex to enable GATA-factor mediated transcription is not yet known.

Dal80p/Uga43p. Dal80p negatively regulates the derepression of various nitrogen catabolic genes that function in a variety of nitrogen catabolic pathways. It allows a low level of derepression of numerous Gln3p-dependent genes, including inducible (*DAL7, DAL4*, *DAL2*, *DUR1,2* and *UGA1*) and constitutive (*DAL3, CAN1* and *GAP1*) genes (Cunningham and Cooper, 1991; Daugherty *et al.*, 1993; Jauniaux and Grenson, 1990; Yoo *et al.*, 1985). Analyses of Dal80p-regulated genes in a *dal80* mutant strain show a vast increase in derepressed transcription and inducer-independent transcription of the inducible *DAL* genes (Cunningham and Cooper, 1991; Daugherty *et al.*, 1993).

Dal80p binds optimally to a DNA binding site consisting of two 5'-GATAA-3' sequences. This was expected, as the DNA binding domains of Gln3p and Dal80p are highly homologous. However, the GATAA sequences must be oriented either tail-to-tail or head-to-tail and separated by 15-30 nucleotides (nt) with 20 nt being the optimal separating distance. This site is needed to regulate the derepression of NCR of many genes and is called the URS_{GATA} (Upstream Repression Sequence containing two GATAA elements) (Cunningham and Cooper, 1993). In addition, Coornaert *et al.* (1992) showed that the ability of Dal80p to bind DNA is essential for the protein to function. A point mutation that changed the highly conserved glycine residue in its C_4 zinc finger to a serine residue abolished Dal80p function.

Arrays of contiguous head-to-tail or tail-to-tail GATAA sequences are common in NCR-sensitive gene promoters that are regulated by Dal80p. These arrays are present in *DAL7*, *DAL3*, *DUR1,2*, *DUR3*, *UGA4*, *CAN1*, *GAP1* and *MEP2*. They are rarely found in the promoters of NCR-sensitive, but Dal80p-independent genes (*DAL5*). These observations strongly suggest that the arrays are responsible for the Dal80p-facilitated regulation of derepression (Rai *et al.*, 1999).

Gln3p and Dal80p bind the same UAS_{NTR}s of *DAL3*, *UGA4*, *GAT1*, and *DAL80* (André *et al.*, 1995; Coffman *et al.*, 1996; 1997; Cunningham *et al.*, 1994). Interestingly, Dal80p (~30 kDa) is much smaller than Gln3p (~80 kDa). This supports the generally accepted theory that these two regulators compete for binding to the same GATAA elements. The outcome of this competition will result in the regulated level of derepression specific to each Gln3p-dependent and Dal80p-regulated gene. If Dal80p indeed regulates derepression solely via competition with Gln3p, it does not need a domain to interact with the core transcription complex (Cunningham and Cooper, 1993).

The requirement for two of these GATAA elements to bind Dal80p was the first indication that this protein might need to dimerize to form a protein-DNA complex (Cunningham and Cooper, 1993). Leucine zipper coiled-coils are protein motifs that enable dimerization in protein complexes (Hu *et al.*, 1995; Pu and Struhl, 1993). Analysis of the Dal80p protein identified a leucine zipper coiled-coil domain, located in the 55 C-terminal amino acid residues, that is essential for Dal80p function (Coornaert *et al.*, 1992). Svetlov and Cooper (1998) showed that Dal80p can homodimerize and also form heterodimers with Deh1p *in vivo*. It is possible that Dal80p will dimerize prior to binding its DNA-binding site.

Three lines of evidence emphasize the complexity of Dal80p-mediated regulation of NCR-sensitive gene expression. Firstly, the mere presence of multiple GATAA elements in a promoter does not constitute Dal80p-mediated regulation. The expression of *DAL5* and *GLN1* are NCR-sensitive and Gln3p-dependent, but not Dal80p-regulated, regardless of the multiple GATAA elements of the respective promoters (Daugherty *et al.*, 1993). The GATAA cores of URS_{GATA} alone will therefore not determine whether or not Dal80p binds to the site. Secondly, the expression of *UGA1* and *PUT2* are NCR-sensitive and regulated by Dal80p, but Gln3p-independent (Cunningham and Cooper, 1991; Daugherty *et al.*, 1993). In addition to its regulation of some Gln3p-dependent genes, Dal80p has also been shown to regulate Gat1p-dependent transcription (Cunningham *et al.*, 2000b). Thirdly, Dal80p contains a dimerization domain necessary for homodimerization and heterodimerization with Deh1p (Svetlov and Cooper, 1998). The significance of heterodimerization is currently not clear. Taken together these observations indicate that the precise mechanism of action of Dal80p depends on the gene it regulates.

Deh1p/Nil2p/Gzf3p. A conserved GATA-type zinc finger domain classifies the fourth GATA factor, Deh1p, as a GATA factor (Stanbrough *et al.*, 1995). The absence of an acidic activation domain, as found in Gln3p and Gat1p, along with the presence of a leucine zipper, relates it to Dal80p (Soussi-Boudekou *et al.*, 1997). Analyses of the phylogenic tree of eukaryotic GATA-type zinc finger proteins show that Deh1p is more closely related to Dal80p than to Gln3p or Gat1p (Coffman *et al.*, 1997).

Deh1p, unlike Dal80p, does not function in derepressive conditions (Coffman *et al.*, 1997; Cunningham and Cooper, 1991; Rowen *et al.*, 1997; Soussi-Boudekou *et al.*, 1997). Instead, it partially represses Gat1p-mediated transcriptional activation in

repressive nitrogen conditions. It is hypothesized that Gat1p and Deh1p competes for binding to similar DNA-binding sites, much as Dal80p and Gln3p does (Coffman *et al.*, 1997; Rowen *et al.*, 1997; Soussi-Boudekou *et al.*, 1997).

An intricate network of interdependent regulation

In addition to the regulatory effects of the GATA factors on the expression of NCR-sensitive genes, the concentration and/or functionality of the four GATA factors are also controlled in response to the nitrogen supply of the cell. All four GATA factors are involved in an intricate network of transcriptional regulation that ultimately regulates the expression of three GATA factor-encoding genes. Extensive cross- and autogenous regulation by the GATA factors exist in specific nitrogen conditions to result in complex interdependent transcriptional regulation (Coffman *et al.*, 1997; Soussi-Boudekou *et al.*, 1997), which ultimately determines the concentrations of these factors in the cell (Fig. 11). The expression of three GATA factor genes, *DAL80*, *GAT1* and *DEH1*, are NCR-sensitive and, consistently, contain multiple GATAA elements in their respective promoter regions (Coffman *et al.*, 1997; Coornaert *et al.*, 1992; Cunningham and Cooper, 1991; Soussi-Boudekou *et al.*, 1997). In contrast, the promoter region of *GLN3* does not contain GATAA elements and its transcription is not nitrogen regulated (Minehart and Magasanik, 1991). However, the functions of Gln3p and Gat1p are regulated by the cell's nitrogen supply. These activators are active in derepressive nitrogen conditions, but largely inactive in repressive nitrogen conditions (Beck and Hall, 1999; Bertram *et al.*, 2000; Cardenas *et al.*, 1999; Cunningham *et al.*, 2000a; Hardwick *et al.*, 1999).

The transcriptional regulation of *DAL80* is the most complex of all the GATA factor genes. It is highly NCR-sensitive (Coornaert *et al.*, 1992; Cunningham and Cooper, 1991). Derepressive nitrogen conditions result in Gln3p- and Gat1p-dependent activation and autogenously regulated repression of *DAL80* transcription (Fig. 11A) (Coffman *et al.*, 1997; Coornaert *et al.*, 1992; Cunningham and Cooper, 1991; Cunningham *et al.*, 2000a; Soussi-Boudekou *et al.*, 1997). Consistently, Gln3p and Dal80p bind to the promoter region of *DAL80* (Coffman *et al.*, 1997). *DAL80* expression results in i) decreased *GAT1* transcription, and consequently Gat1p-mediated transcription, and ii) repression of Gln3p-dependent transcriptional activation. Dal80p thereby provides a feedback loop that fine-tunes the abundance of Gat1p and the

functions of both positive regulators in derepressive nitrogen conditions (Cunningham *et al.*, 2000b; Daugherty *et al.*, 1993). In repressive nitrogen conditions, *DAL80* transcription decreases due to the cytoplasmic retention of Gln3p and Gatlp, which involves Ure2p (Beck and Hall, 1999; Bertram *et al.*, 2000), and the repressive effect of Dehlp (Fig. 11B) (Coffman *et al.*, 1997; Soussi-Boudekou *et al.*, 1997). Thus, both the negative regulators, Ure2p and Dehlp, are required to repress *DAL80* transcription in repressive nitrogen conditions. The regulation of *DAL80* transcription is therefore mediated by all the GATA factors, including its own gene product, to ensure the proper ratio of the different GATA factors to optimally regulate NCR-sensitive genes in different nitrogen conditions (Coffman *et al.*, 1997; Soussi-Boudekou *et al.*, 1997).

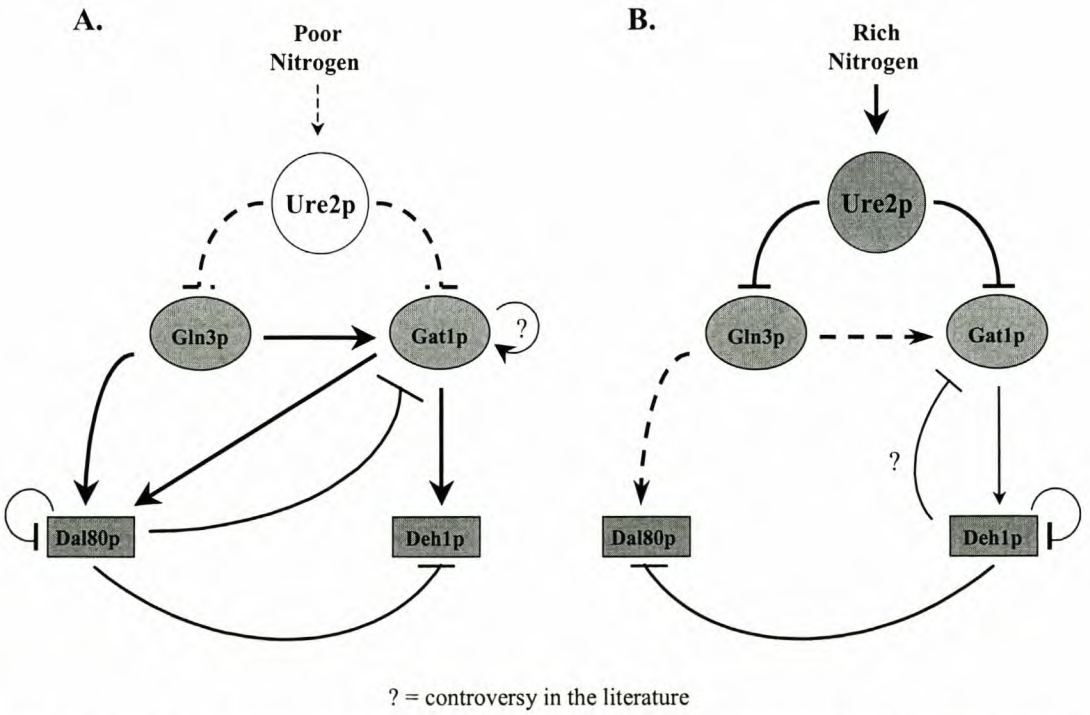


Fig. 11. Interdependent regulation of nitrogen-regulated transcriptional regulators in correlation with the nitrogen supply.

GAT1 expression is NCR-sensitive, Dal80p-repressed and partially Gln3p-dependent. Both these regulators bind to the promoter region of *GAT1* (Fig. 11A). In addition, the transcription of *GAT1* is modestly activated independent of Gln3p in derepressive nitrogen conditions (Coffman *et al.*, 1996). To this end, Rowen *et al.* (1997) reported that *GAT1* transcription is autogenously activated by its gene product. In contrast, Coffman *et al.* (1997) reports that *GAT1* is transcribed independent of

Gat1p. A yet unknown activator could therefore be involved in the Gln3p-independent transcription of *GAT1*.

GAT1 expression and Gat1p function are tightly linked to the concentration and function of Ure2p (Beck and Hall, 1999; Cunningham *et al.*, 2000a). Ure2p is active in repressive nitrogen conditions and inhibits the activity of Gln3p (Beck and Hall, 1999; Bertram *et al.*, 2000; Cardenas *et al.*, 1999; Hardwick *et al.*, 1999), thereby providing a rough mechanism to partially decrease the expression of *GAT1* when its gene product is less needed. In addition, Deh1p also down-regulates the expression of *GAT1* in repressive conditions (Fig. 11B) (Rowen *et al.*, 1997). In contrast, Coffman *et al.* (1997) reported that Deh1p does not repress *GAT1* expression.

DEH1 expression is modestly NCR-sensitive, modestly Gat1p-dependent, highly Dal80p-repressed, and largely Gln3p-independent (Fig. 11A) (Coffman *et al.*, 1997; Soussi-Boudekou *et al.*, 1997). *DEH1* is expressed several-fold less than *DAL80* (Coffman *et al.*, 1997; Soussi-Boudekou *et al.*, 1997) and its gene product has a very limited effect on Gln3p-mediated transcription (Rowen *et al.*, 1997). In repressive nitrogen conditions Deh1p represses its own transcription, thus performing an autogenous regulatory function just like Dal80p does (Fig. 6B) (Soussi-Boudekou *et al.*, 1997). This is an interesting phenomenon as *DEH1* expression decreases in repressive nitrogen conditions, thereby decreasing the concentration of its gene product in the cell. Yet it is in these repressive conditions that the protein performs its major role in the regulation of Gat1p-dependent gene expression. The precise reason for this is not yet clear.

Phenotypically the *ure2* and *deh1* mutants share similarities; both mutants relieve nitrogen-related repression on some, but not all, nitrogen-regulated genes in repressive conditions (Coffman *et al.*, 1994; 1997; Soussi-Boudekou *et al.*, 1997). Two observations question whether or not nitrogen-mediated repression is the major function of Deh1p. Firstly, the expression of *DEH1* is modestly NCR-sensitive and consequently decreases in repressive nitrogen conditions, when the protein has been shown to function (Coffman *et al.*, 1997; Soussi-Boudekou *et al.*, 1997). Secondly, Deh1p has only been shown to affect a very limited number of nitrogen-regulated genes (Coffman *et al.*, 1997; Soussi-Boudekou *et al.*, 1997).

GLN3, which encodes a strong activator, is constitutively expressed in the cell. In repressive conditions it is restricted to the cytoplasm and cannot activate transcription of its target genes (Fig. 9A). However, when cells are moved to derepressive nitrogen conditions, Gln3p is released from its complex with Tor1p and Ure2p and it translocates to the nucleus to activate transcription of its target genes (Fig. 9B) (Beck and Hall, 1999; Bertram *et al.*, 2000). This initial transcriptional activation is not regulated as i) Deh1p does not repress Gln3p-mediated transcription and ii) *DAL80* transcription is repressed by NCR. Dal80p is consequently not present to repress Gln3p-mediated activation. However, one of the Gln3p target genes is *DAL80*. Thus, to counter the large initial transcriptional activation by Gln3p, a large amount of its repressor, Dal80p, is produced (Fig. 11A). The expression of *DAL80* inhibits Gln3p-dependent transcriptional activation (Cunningham *et al.*, 2000b).

The controlled interdependent expression of the GATA factor genes can function to ensure that these factors are synthesized at proper concentrations in correlation with the nitrogen supply of the cell. In derepressive nitrogen conditions the activities of the positive regulators and Dal80p are high, resulting in diminished activity of Deh1p. The situation reverses in repressive nitrogen conditions when Deh1p is rendered active and the activators and Dal80p largely inactive.

Induction

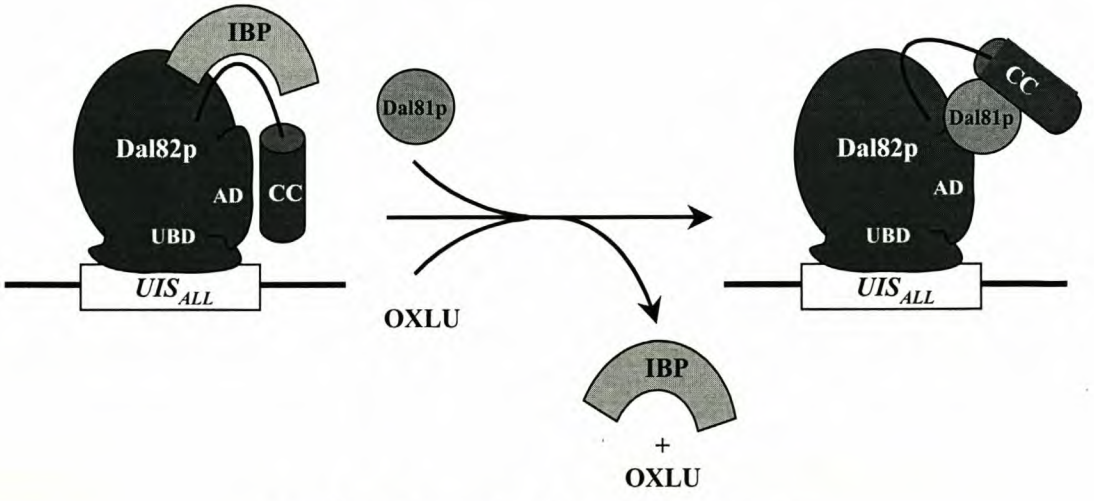
The maximum levels of transcription of the *DAL* and *DUR* genes can be achieved in either an inducer-independent (constitutive) or inducer-dependent (induced) manner. Inducer-independent transcriptional activation is mainly achieved by GATA factor-dependent derepression of NCR alone. However, allophanate (or OXLU) serves as an inducer molecule that enhances the transcription of the inducible *DAL* and *DUR* genes following derepression (Cooper, 1994; Cooper *et al.*, 1989; Sumrada and Cooper, 1974). Induction of these genes is therefore superimposed on derepression. In contrast to general transcriptional activation by the derepression of NCR, induction is a pathway-specific response that only affects the transcription of certain genes associated with a specific pathway.

The induced expression of the *DAL* genes involves the relief of Dal80p-dependent regulation of derepression in response to the presence of the inducer (Cunningham and Cooper, 1991; Rai *et al.*, 1999). Induction requires at least three known components:

the pathway inducer allophanate (or OXLU) (Cooper and Lawther, 1973; Sumrada and Cooper, 1974), two known regulatory proteins, Dal81p/Uga35p/DurLp and Dal82p/DurMp (Cooper, 1994) and at least one specific *cis*-acting element, the UIS_{ALL} (ALLophanate-responsive Upstream Induction Sequence) (Yoo and Cooper, 1989).

Dal82p and Dal81p

Dal82p is a relatively small protein (29 kDa), which localizes to the nucleus. It contains three known domains: a UIS_{ALL} DNA-binding domain (aa 1-85), a transcriptional activation core domain (aa 66-99) and a coiled-$coil_{Dal82p}$ motif (aa 217-255) (Fig. 12). The DNA-binding domain has no defined structure, but shares limited homology with insect homeodomain proteins (Scott *et al.*, 2000a). Homeodomains are well known DNA-binding motifs (Gehring *et al.*, 1994). The DNA-binding and transcriptional activation domains overlap and function independently of the coiled-$coil_{Dal82p}$ motif. The latter motif plays a major role in regulating Dal82p-dependent transcription and is proposed to receive most of the inducer signal (Scott *et al.*, 2000b).



OXLU = Oxalurate; IBP = Inducer Binding Protein;
AD = Activation domain; UDB = UIS_{ALL} Binding Domain;
CC = Coiled-$coil_{Dal82p}$

Fig. 12. A model for the interplay between Dal82p, IBP and Dal81p in response to the presence of the inducer (adapted from Scott *et al.*, 2000b). OXLU = the inducer oxalurate; IBP = Inducer Binding Protein; AD = Activation Domain; UBD = UIS_{ALL} Binding Domain; CC = Coiled-$coil_{Dal82p}$.

Dal81p is the second transcriptional regulator needed for UIS_{ALL}-mediated induced expression of the *DAL* genes (Bricmont and Cooper, 1989). This protein has a $Zn(II)_2Cys_6$ DNA binding motif similar to those present in the Cys_2-Zn_2 family of transcription factors, as well as two polyglutamine stretches that are present in the transcriptional activation domains of various eukaryotic transcription factors. Interestingly, deletion of the Dal81p $Zn(II)_2Cys_6$ DNA binding motif did not alter the proteins' ability to support UIS_{ALL}-mediated induction. However, deletion of one of the polyglutamine stretches resulted in a 50% decrease in transcriptional induction (Bricmont *et al.*, 1991).

In the absence of the inducer, the Dal82p-mediated transcriptional activation is down-regulated by the coiled-$coil_{Dal82p}$ motif. However, in the presence of the inducer molecule, Dal81p enables induced transcription via the coiled-$coil_{Dal82p}$. The latter motif supposedly masks the Dal82p activation domain in the absence of the inducer, thereby preventing interaction of the activation domain with the core transcription apparatus and consequently preventing induction. In the presence of the inducer, Dal81p interacts with the coiled-$coil_{Dal82p}$ domain and supposedly unmasks the transcriptional activation domain of Dal82p (Fig. 12). However, Dal81p is not unique in its ability to relieve the coiled-$coil_{Dal82p}$-facilitated repression as some induced transcription still occurs in the *dal81* mutant. An unidentified inducer binding protein (IBP) is proposed to be involved in this repression (Scott *et al.*, 2000b).

cis-Acting element

Dal82p was originally thought to be a pathway-specific transcriptional inducer protein for the *DAL* system as the *dal82* mutants were unable to induce the expression of *DAL7* in response to OXLU (Olive *et al.*, 1991). It binds specifically to the UIS_{ALL} (Dorrington and Cooper, 1993), which has the consensus sequences of 5'-$N^{G}/_{C}AAA^{A}/_{T}NTGCG^{T}/_{C}T^{T/C}/_{G}N$-3' (van Vuuren *et al.*, 1991). This element is needed for the induction of all inducible *DAL* genes and one or more copies are present in the promoters of these genes (Olive *et al.*, 1991; van Vuuren *et al.*, 1991; Yoo and Cooper, 1989). However, Dal82p is also required for the induction of the *CAR2* gene (encodes arginase) and it is therefore also involved in the expression of the arginine catabolic pathway genes (Park *et al.*, 1999).

The two *DAL* genes that show the greatest response to the presence of the inducer, *DAL7* and *DAL4*, contain multiple copies of the UIS_{ALL} element in their respective promoters (Yoo and Cooper, 1989; Yoo *et al.*, 1992). In each promoter, one of these elements shares a greater deal of sequence similarity with the UIS_{ALL} consensus sequence than the remaining copies (van Vuuren *et al.*, 1991). In each case Dal82p binds the more conserved sequence with a higher affinity. Also, of all the inducible *DAL* genes, Dal82p binds the promoters of the most inducer-responsive genes, *DAL4* and *DAL7*, with the highest affinity (Dorrington and Cooper, 1993). Mutation of the *DAL7* UIS_{ALL} elements result in decreased abilities to i) activate transcription in derepressive conditions (Rai *et al.*, 1999) and ii) induce transcription in response to OXLU (van Vuuren *et al.*, 1991).

Dal81p does not function in a pathway-specific manner. The transcriptional induction of many genes from different pathways requires a functional Dal81p. These include genes involved in allantoin and urea degradation (*DAL7* and *DUR1,2* – induced by allophanate/OXLU) (Bricmont and Cooper, 1989), 4-aminobutyrate catabolism (*UGA1*, *UGA2* and *UGA4* – induced by GABA) (Bricmont *et al.*, 1991; Vissers *et al.*, 1989; 1990) and arginine catabolism (*CAR2* – induced by arginine/OXLU) (Hennaut, 1981; Park *et al.*, 1999). In addition, leucine induction of *AGP1* (encoding the broad-specificity amino acid permease) is also Dal81p-dependent (Iraqui *et al.*, 1999). Ssy1p senses extracellular leucine and activates a signal transduction pathway, which ultimately requires Dal81p to induce the transcription of *AGP1*. The diversity of the Dal81p target genes argues that it does not induce transcription in an inducer-specific manner. Also, it does not respond to the signal from only one sensor. The *ssy1* mutant strain does not affect the induction of the *DAL* genes and *AGP1* induction is independent of Dal82p. Thus, Dal81p is a positive general transcriptional regulator whose function is controlled by different sensors.

In an effort to understand the cross talk between the UIS_{ALL} and UAS_{NTR} elements, these elements were analyzed in heterologous expression systems. A single *DAL7* UIS_{ALL} element alone was unable to activate or induce heterologous gene expression in response to either nitrogen derepression or induction conditions, respectively (Yoo and Cooper, 1989). Also, transcriptional activation mediated by a single *DAL7* UAS_{NTR} was not greatly affected by the loss of either Dal81p (Bricmont and Cooper, 1989) or Dal82p (Olive *et al.*, 1991). However, when the three elements UIS_{ALL}, UAS_{NTR}, and

URS_{GATA} were combined (UIS_{ALL}-UAS_{NTR}-URS_{GATA}), Dal82p was needed for both derepression and induction (Olive *et al.*, 1991). Several investigators have subsequently reported the involvement of the UIS_{ALL} in transcriptional derepression (Park *et al.*, 1999; Rai *et al.*, 1999). Also, a UIS_{ALL} placed close to a mutated UAS_{NTR} suppress the latter mutations (van Vuuren *et al.*, 1991). The UIS_{ALL} and Dal82p are therefore not only required for induction, but also for the derepression of the inducible *DAL* genes (Olive *et al.*, 1991; Rai *et al.*, 1999).

Collectively these data led to the hypothesis that the UIS_{ALL}-Dal82p complex influences UAS_{NTR}-mediated activation. The complex was proposed to enhance the function of Gln3p and/or Gat1p either directly via protein-protein interactions between Dal82p and Gln3p/Gat1p (Rai *et al.*, 1999; van Vuuren *et al.*, 1991), or indirectly via components of the core transcription apparatus (Park *et al.*, 1999). Scott *et al.* (2000b) observed that Dal82p and Gln3p have different target sites in the SAGA complex. The authors propose that the functional interaction of these two transcriptional activators is due to their respective unique protein-protein interactions with the SAGA complex.

Despite the requirement of Gln3p and/or Gat1p for Dal82p-mediated transcriptional induction of the *DAL* genes, Dal82p can function independent of these activators. *CAR2* is induced by arginine via the ArgR proteins and by allophanate/OXLU via the Dal82p (Messenguy *et al.*, 1991; Park *et al.*, 1999). Rap1p and Abf1p are required for the transcriptional activation of *CAR2*, independent of the nitrogen source supplied (Park *et al.*, 1999). This activation is balanced by the action of the repressor Ume6p (Sumrada and Cooper, 1987). The presence of an inducer shifts this balance towards activation and the absence thereof towards repression. Rap1p, Abf1p and Dal82p bind to the promoter of the *CAR2* gene. Rap1p and Dal82p strongly bind their respective DNA-binding sites, while Abf1p only binds with limited affinity. These binding sites are needed for the maximal expression of *CAR2*. The *CAR2* promoter is devoid of GATAA elements. In this case Rap1p has replaced the GATA factors needed for the activated expression of other nitrogen-regulated genes, indicating that Dal82p can function independent of the GATA factors (Park *et al.*, 1999). This is in contrast to the observation for the *DAL* system genes where Dal82p enhances the action of the GATA factors (Rai *et al.*, 1999; van Vuuren *et al.*, 1991). In addition, Dal82p has the ability to activate *CAR2* transcription independent of Dal81p and the inducer molecule OXLU (Park *et al.*, 1999).

The specific quantities of Dal81p and Dal82p in the nucleus can also play a role in the expression of the inducible *DAL* genes. Although the transcriptional regulation of the *DAL82* gene is not as dramatic as the permease- and enzyme-encoding *DAL* genes, it is NCR-sensitive and Dal80p- and Dal81p–regulated. Interestingly, *DAL82* expression increases in both the *dal80* and *dal81* mutant strains when these cells were grown in either derepressive or inducible conditions. It is unexpected that Dal81p down-regulates the expression of *DAL82*, although the repressive phenotype of the *dal80* mutant was expected. In addition, the derepression of *DAL82* is not solely dependent on the Gln3p. The level of *DAL82* expression only decreases by 30% in a *gln3* mutant strain grown in derepressive conditions compared to the wild type (Olive *et al.*, 1991). Other transcriptional activators involved in *DAL82* derepression have not yet been identified. In contrast, the expression of the *DAL81* gene is neither NCR-sensitive nor inducible. Interestingly, its expression is modestly Dal80p-regulated (Bricmont and Cooper, 1989).

A new *cis*-acting element involved in the induction of the *DAL7* gene has been reported. A GC-rich inverted repeat with the sequence 5'-CCGCGG-3' is located between the two UIS_{ALL} elements in the *DAL7* promoter. Mutation of this sequence affects heterologous gene expression in two ways: firstly, derepressed and induced levels of expression are severely decreased upon mutation and secondly, the ability to respond to the inducer (fold induction) increased. However, when the mutated element was studied in the context of the full-length *DAL7* promoter, the effect on transcription was not as dramatic as with the cloned heterologous fragment. Derepression and induction were only mildly affected. Nonetheless, this element shows that a second mechanism, in addition to the UIS_{ALL}-Dal82p-mediated enhancement of Gln3p and/or Gat1p activation, exists for the induction of *DAL7* (Rai *et al.*, 1999). A similar positively acting GC-rich element has also been reported for the *CAR1* gene (Smart *et al.*, 1996). However, it is not yet clear whether these elements have the same function or bind similar proteins. Although the exact mechanism of action of this element is currently unclear, it does seem to function synergistically with the Gln3p and/or Gat1p (Rai *et al.*, 1999).

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RESEARCH PAPERS

CHAPTER 3

Cis-acting Sites Contributing to Expression of Divergently Transcribed *DAL1* and *DAL4* Genes in *S. cerevisiae*: A Caveat When Correlating *cis*-Acting Sequences With Genome-Wide Expression Analyses

G.K. van der Merwe, T.G. Cooper, and H.J.J. van Vuuren

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Cis-acting Sites Contributing to Expression of Divergently Transcribed *DAL1* and *DAL4* Genes in *S. cerevisiae*: A Caveat When Correlating *cis*-Acting Sequences With Genome-Wide Expression Analyses

George K. van der Merwe[1], Hennie J.J. van Vuuren[1], and Terrance G. Cooper[2]

[1]B.C. Wine Research Center, Faculty of Agricultural Sciences, University of British Columbia, Vancouver, BC V6T 1Z4, Canada, and the [2]Department of Microbiology and Immunology, University of Tennessee, Memphis, TN 38163, U.S.A.

Abstract. Correlating genome-wide expression profiles with sequence searches of promoter regions is being used as a technique to identify putative binding sites for *trans*-acting factors or to refine consensus sequences of those already known. To evaluate the limitations of such an approach in our studies of GATA-mediated transcription in *Saccharomyces cerevisiae*, we identified the relative contributions made to *DAL1* and *DAL4* expression by each of five Gln3p-, and/or Gat1p-, and three Dal82p-binding site homologous sequences situated in the 829 bp intergenic region separating these highly related, divergently transcribed genes. Our data suggest that although correlations of repeated sequences or sequence homologies appearing within promoter regions with expression profiles obtained from genome-wide transcription analyses provide useful starting points for analyses of *cis*-acting sites, significant limitations and possibilities for misinterpretation also abound.

Introduction

Cluster analysis of genome-wide gene expression data is currently being used to identify genes controlled by known transcription factors and to identify and generate consensus sequences for transcription factor-binding sites (Kruglyak and Tang, 2000; Lyons et al. 2000). Such analyses usually designate 1 kb upstream of the clustered gene's ATG as the search target, following the rationale that "two genes that are controlled by a single regulatory system should have similar expression patterns in any data set" (Kruglyak and Tang, 2000). Although such analyses can yield useful information, they are also potentially subject to significant problems and limitations, particularly in cases of

divergently transcribed genes sharing small intragenic regions. The principal difficulties are knowing whether sequences, observed by homology or repeated appearance in an upstream region, are: (1) functioning in vivo, (2) shared equally by the divergently transcribed genes, and (3) influenced by the operation of other *trans*-acting factors.

A brief survey of the literature yielded analyses of 14 divergently transcribed genes (Angermayr and Bandlow 1997;Bell et al. 1995, 1997; Friesen et al. 1997; Hahn et al. 1988; Halfter et al. 1989; Johnston and Davis 1984; Kraakman et al. 1989; Kruglyak and Tang 2000; Liu and Xiao 1997; Osley et al. 1986; Schlapp and Rodel 1990; Siliciano and Tatchell 1984; Struhl 1985; Thuriaux et al. 1995); and the genome contains many more that are unstudied. In most cases, analyses identified relatively large DNA fragments (greater than 40-100 bp) that support regulated gene expression in heterologous vectors. For a few (e.g., *GAL1-GAL10*, *PET56-HIS3*, *MAL6T-MAL6S*), the analyses have been more comprehensive although not exhaustive (Bell et al. 1995; Johnston and Davis, 1984; Struhl, 1985;). For example, *GAL1* and *GAL10* are reported to be regulated in common by four Gal4p-binding sites situated in a 75-bp region between them (West et al. 1984).

To evaluate problems potentially associated with making correlations such as those mentioned above, we analyzed, as a model, the expression of two closely-related, divergently transcribed genes, *DAL1* and *DAL4* (encoding allantoinase and allantoin permease, respectively; Cooper 1996). They share an intergenic region (829 bp) roughly the same size as *GAL1-GAL10* (680 bp) and eight sequences qualifying as homologous to known allantoin pathway transcription factor-binding sites.

Two types of *cis*-acting elements are responsible for regulated *DAL* gene expression (see Cooper 1996; Hofman-Bang 1999; ter Schure et al. 2000; Wiame et al. 1985 for comprehensive reviews of the GATA-transcription factor literature): (1) upstream activating sequence (UAS) UAS_{NTR} elements and (2) upstream induction sequence (UIS) UIS_{ALL} elements. UAS_{NTR} elements are dodecanucleotides with the sequence GATAA at their core (Bysani et al. 1991) that are binding sites both for the transcriptional activators Gln3p and/or Gat1p/Nil1p (Blinder and Magasanik 1995; Cunningham et al. 1996) and for the competing GATAA-binding repressor protein Dal80p (Cunningham and Cooper 1993; Cunningham et al. 1994). UIS_{ALL} elements are dodecanucleotides that are binding sites for Dal82p which, along with other protein(s), are responsible for allophanate-induced gene expression (Dorrington and Cooper 1993; van Vuuren et al.

1991). The ability of two of the UIS_{ALL} elements situated between *DAL1* and *DAL4* to bind Dal82p has been measured (Dorrington and Cooper 1993). The inducer of *DAL* gene expression is allophanate, the last intermediate in the pathway or its non-metabolized analogue oxalurate (OXLU; Cunningham and Cooper 1993). There are five UAS_{NTR}-homologous and three UIS_{ALL}-homologous sequences in the *DAL1-DAL4* intergenic region that would qualify for inclusion in genome-wide correlations such as those mentioned above.

In this work, we determined the relative contributions of each UAS_{NTR}- and UIS_{ALL}-homologous sequence in the *DAL1-DAL4* intragenic region. The information obtained argues that even when analyzing highly related, divergently transcribed genes, whose transcription is supported by well-characterized *cis*-acting elements, it is difficult to draw rigorous conclusions about: (1) which of the *cis*-acting element-homologous sequence(s) found in a promoter region are actually responsible for the observed transcription, (2) their relative contributions to the overall transcription profile, and (3) the significance that should be attached to their presence in generating/refining a consensus sequence or identifying candidate sequences mediating an observed form of regulation in the absence of detailed biochemical analysis.

Materials and methods

Strains and media

All *Saccharomyces cerevisiae* strains used in this research are isogenic derivatives of the wild-type TCY1: TCY1 (*Matα ura3 lys2*), TCY17 (*Matα ura3 lys2 dal80Δ::hisG*), RR91 (*Matα ura3 lys2 gln3Δ::hisG*), HEY6 (*Matα ura3 lys2 dal81Δ::hisG*), and SS400 (*Matα ura3 lys2 trp1 dal82Δ::TRP1*). We also used *E. coli* strain *DH5α* F'/endA1 hsdR17($r_K^- m_K^+$) supE44 thi1 recA1 gyrA (*Nal*^{r}) relA1 Δ(lacZYA-argF)$_{U169}$ (m80lacZΔM15). Yeast cultures for β-galactosidase and Northern blot analyses were grown in yeast nitrogen base (YNB) medium (0.17% YNB without amino acids and ammonium sulfate; Difco Laboratories, Detroit, Mich.), supplemented with 2% glucose, amino acids required to complement auxotrophies, and either 0.1% glutamine (repressed) or proline (derepressed) as sole nitrogen source. Gratuitous inducer, OXLU, was added to proline-containing medium (final concentration, 0.5 mM).

Plasmid construction and PCR

A *CEN*-based *lacZ* reporter plasmid, for analysis of expression supported by wild-type and mutant alleles of the *DAL1-DAL4* intergenic promoter region, was constructed by cloning a *Bam*HI-linker (5'-CGCGGATCCGCG-3') into the *Sma*I site of pHP41 (Park et al. 1992) to yield pVAN1. *Bam*HI digestion of pVAN1 yielded a 10 kb fragment which was isolated and re-circularized to yield pVAN2, which served as the parent plasmid in all β-galactosidase assays.

PCR-based methods were used to create deletion and substitutions mutations in the *DAL1-DAL4* intergenic promoter region. Primers GK2 (5'-GCGCGGATCCGGAAGTGATGGCATTGATAGGCATC–3'), and GK3 (5'-GCGCGGATCCAGCACTTAGAGCGTCGTTAGCCATT–3') were used to synthesize a fragment covering nucleotides from +24 *DAL1* to *DAL4* +24, thereby allowing in-frame fusion of either the *DAL1* ATG or *DAL4* ATG to the *lacZ* gene of pVAN2. The other primers we used are listed in Table 1.

The strategies used to mutate a specific potential *cis*-acting element, template and primer combinations used, and the plasmids created are presented in Table 2. Site-directed mutations were constructed according to Viljoen et al. (1999). Heat-stable DNA polymerase PWO (Roche Molecular Biochemicals) was used in all PCR reactions. Reaction conditions and amplification programs were as prescribed by the manufacturer. All PCR products were digested with *Bam*HI and cloned into pVAN2. The integrity of all DNA fragments synthesized by PCR and in-frame fusions was confirmed by sequence analysis. The mutations introduced into each mutated promoter construct are listed in Table 2.

Yeast and bacterial transformation

Yeast (Geitz et al. 1992) and bacterial (Inoue et al. 1990) transformation procedures have been described previously.

β-Galactosidase assays

β-Galactosidase assays were performed essentially as described (Smart et al. 1996), except that we analyzed 10 ml of culture instead of 25 ml. Assays were performed in duplicate and from at least two independent yeast transformations. Data from duplicate assays generally varied less than 5% and of repeated transformations less than 20%.

Enzyme activities are expressed in Miller units (Miller 1972), but are based on 10 ml of culture.

Table 1 Oligonucleotides used in this research. Applications are relative to *DAL1* ATG (+1), unless otherwise indicated. *Italics* indicate mutations introduced. *Bold* indicates restriction sites used for cloning and introduction of mutations

Primer	Sequence	Application
GK2	5'-GCGC**GGATCC**GGAAGTGATGGCATTGATAGGCATC–3'	*DAL1*-ATG *lacZ* fusion
GK3	5'-GCGC**GGATCC**AGCACTTAGAGCGTCGTTAGCCATT–3'	*DAL4*-ATG *lacZ* fusion[a]
Deletion analysis		
GK7	5'-GCGC**GGATCC**GGGACAATAGAATCGAAACATGC-3'	-543 of *DAL4* ATG[a]
GK8	5'-GCGC**GGATCC**AGCGGTCAATCCATCCTATTA-3'	-560 of *DAL1* ATG
GK20	5'-GACT**GGATCC**CTGCATGTTTCGATTCTATT-3'	-307 of *DAL1* ATG
UAS_{NTR} site-directed mutation analysis		
GK25	5'-TTGCGGTGCTTA**GACGTC**TATATAGAGGAG –3'	Mutate -188 to -193
GK26	5'-CTCCTCTATATA**GACGTC**TAAGCACCGCAA –3'	Mutate -188 to -193
GK11	5'-ACC**AAGCTT**AGATACCCTCGAGCTGCATGT-3'	Mutate -357 to -362
GK12	5'-TCT**AAGCTT**GGTATTACTTTCTTATCAATG-3'	Mutate -357 to -362
GK13	5'-TT**CCATGG**AAAGTAATACCGATAAGAGATA-3'	Mutate -384 to -389
GK14	5'-TT**CCATGG**AATGAAAAATTTCTGCCAGGGA–3'	Mutate -384 to -389
GK15	5'-TC**CAATTG**GCAACTAGATTAGAGGCGCTAT–3'	Mutate -474 to -479
GK16	5'-GC**CAATTG**GAATGTGTATGTGTAATTGAAG–3'	Mutate -474 to -479
GK17	5'-AC**GAATTC**GACGTGACAGCAAAGCGGTCAA-3'	Mutate -574 to -579
GK18	5'-TC**GAATTC**GTTCCTTTAAAGATTGTGTCCA-3'	Mutate -574 to -579
UIS_{ALL} site-directed mutation analysis		
GK33	5'-GATC*ACTAG***CAATTG**GCTTAATTATCTATATAGAGG-3'	Mutate -197 to -210
GK34	5'-GATC**CAATTG***CTAGT*GAACCACTTCTCCTGATTAAG-3'	Mutate -197 to -210
GK37	5'-GATC**GAATTC***ATATG*CCCTGGCAGAAATTTTTCATT-3'	Mutate -399 to -411
GK38	5'-GATC*CATAT***GAATTC**GCTTTTTTTCCGGCCATCCTTA-3'	Mutate -399 to -411
GK41	5'-GATC*TGATCA***ATGCAT**GTATGCGACAGCGAGTAAG-3'	Mutate -448 to -460
GK42	5'-GATC**ATGCAT***TGATCA*CTAATCTAGTTGCGATAAGG-3'	Mutate -448 to -460

[a]Relative to *DAL4* ATG (+1)

Table 2. PCR strategies to construct various deletion and substitution mutations in the *DAL1-DAL4* intergenic region. Sequence coordinates are relative to the *DAL1* ATG

Sequences analyzed[a]	**Template & primers for PCR**	**Mutation**	**Plasmids created DAL1 & DAL4**
Wild-type promoter	pTC12[a]; GK2 and GK3	None	pGV1 & pGV2
UAS_{NTR} directed mutations			
GATA1 (-188 to –193)	pTC12; GK2/GK25 & GK26/GK3	ATTATC → gacgTC	pGV3 & pGV4
GATA2 (-357 to –362)	pTC12; GK2/GK11 & GK12/GK3	GATAAG → aAgctt	pGV5 & pGV6
GATA3 (-384 to –389)	pTC12; GK2/GK13 & GK14/GK3	GATAAG → ccatgG	pGV7 & pGV8
GATA4 (-474 to –479)	pTC12; GK2/GK15 & GK16/GK3	CTTATC → CaatTg	pGV9 & pGV10
GATA5 (-574 to –579)	pTC12; GK2/GK17 & GK18/GK3	CTTATC → gaatTC	pGV11 & pGV12
GATA2 & 3	pGV8[b]; GK2/GK11 & GK12/GK3	Combine *gata2 & 3* mutations	pGV13 & pGV14
GATA4 & 5	pGV12[b]; GK2/GK15 & GK16/GK3	Combine *gata4 & 5* mutations	pGV15 & pGV16
UIS_{ALL} directed mutations			
UIS6 (-197 to –210)	pTC12; GK2/GK33 & GK34/GK3	CAAAATTGCGGTGC → CActAgcaattgGC	pGV21 & pGV22
UIS7 (-399 to –411)	pTC12; GK2/GK37 & GK38/GK3	GGGCGCATTTTCC → GaattcaTaTgCC	pGV27 & pGV28
UIS8 (-448 to –460)	pTC12; GK2/GK41 & GK42/GK3	AGGCGCTATTTTG → tGatcaatgcaTG	pGV33 & pGV34
	pGV22[b]; GK2/GK37 & GK38/GK3	Combine *uis6 & uis7* mutations	pGV35 & pGV36
	pGV34[b]; GK2/GK37 & GK38/GK3	Combine *uis7 & uis8* mutations	pGV37 & pGV38
	pGV34; GK2/GK33 & GK34/GK3	Combine *uis6 & uis8* mutations	pGV39 & pGV40
	pGV22; GK2/GK37 & pGV34; GK3/GK38	Combine *uis6, uis7 & uis8* mutations	pGV41 & pGV42
Deletion mutations			
DAL4 promoter	pTC12; GK2/GK7	Delete 286 bp of the *DAL4* promoter	pGV45
DAL1 promoter	pTC12; GK2/GK8	Delete 269 bp of the *DAL1* promoter	pGV46
DAL1 promoter	pTC12; GK2/GK20	Delete 522 bp of the *DAL1* promoter	pGV47

[a] Buckholz and Cooper (1991)
[b] This work

Northern blot analysis

Total RNA was isolated from cultures grown to mid-log phase (A_{600} = 1.0; Ausubel et al. 1994). Poly(A)$^+$ RNA was isolated using the PolyATtract mRNA isolation system III (Promega), according to the manufacturer's recommendations. Samples of Poly(A)$^+$ RNA were resolved on 1.2 % agarose-formaldehyde gels and transferred to Genescreen Plus 66 nylon membranes (NEN Research Products, Dupont). Double-stranded DNA probes used in the Northern blot analyses were synthesized by PCR using the oligonucleotides DAL1-5 (5'-CTGGCATCAATGAAAGC-3') and DAL1-3 (5'-CTGCAGCAATACACAAA-3') for *DAL1*, DAL4-5 (5'-ATGGCTAACGACGCTCT-3') and DAL4-3 (5'-TATGACACAATAGATGT-3') for *DAL4*, and H4-5 (5'-GGCCGGATCCATGTCCGGTAGAGGTAAAGG-3') and H4-3 (5'-GGCCGAATTCTTAACCACCGAAACCGTATAAGG-3') for *H4*. DNA probes were radioactively labeled by random priming (Roche Molecular Biochemicals). Standard prehybridization, hybridization and washing conditions were followed (Ausubel et al. 1994).

Results

Steady-state *DAL1* and *DAL4* expression (mRNA) profiles

To establish the basic *DAL1* and *DAL4* expression profiles, we analyzed steady-state RNA from wild-type strain TCY1, grown in glucose-proline medium with and without OXLU. Although an 829-bp intergenic region is shared by *DAL1* and *DAL4*, the genes are regulated differently. *DAL4* is much more inducer-responsive than *DAL1* (Fig. 1), as reported earlier on the basis of enzyme activities in Σ1278b-based strains.

To identify the *cis*-acting elements that mediate transcription of the two genes, we constructed in-frame *DAL1-* and *DAL4-lacZ* fusions pGV1 and pGV2, respectively. Deletion of the *DAL4* third of the intergenic region (pGV46) had little effect on *DAL1* expression; and a larger deletion eliminated all *DAL1* expression (pGV47; Fig. 2). Deletion of the *DAL1* third (pGV45) decreased induced *DAL4* expression by 2/3 (Fig. 2). All *DAL1-lacZ* and *DAL4-lacZ* expression was highly nitrogen catabolite repression (NCR)-sensitive, Gln3p-dependent, and Dal81p-dependent (Fig. 2 and data not shown). Induced β-galactosidase production from *DAL1-lacZ* and *DAL4-lacZ* differed by 15-fold.

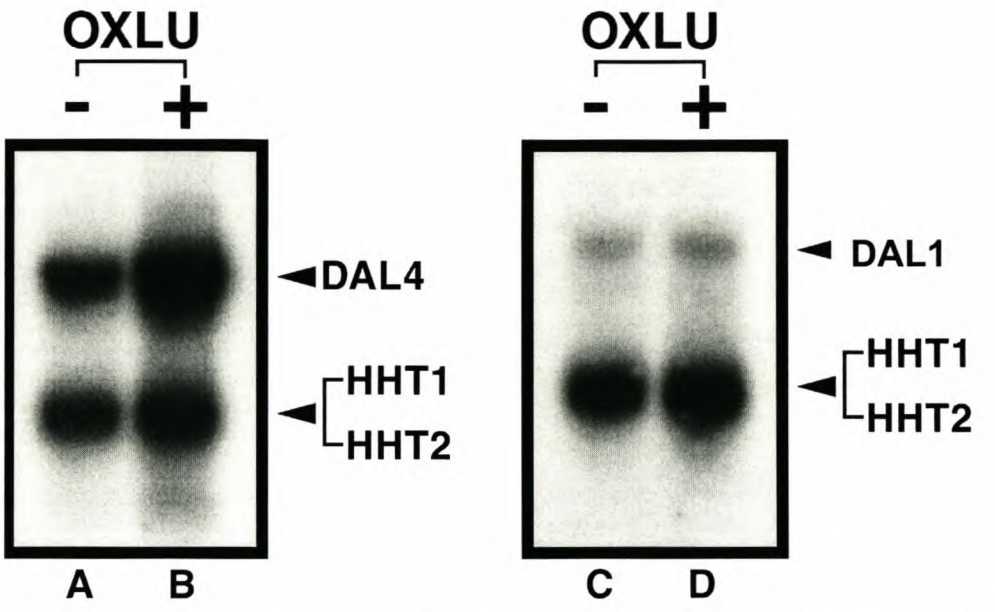

Figure 1. *Saccharomyces cerevisiae DAL1* and *DAL4* expression in the presence (*lanes B, D*) and absence (*lanes A, C*) of the allantoin pathway inducer oxalurate (*OXLU*). Poly(A)$^+$ RNA was prepared from wild-type strain TCY1 grown in glucose-proline yeast nitrogen base (YNB) media with (+) or without (-) 0.5 mM OXLU. Histone H4 served as a control for mRNA loadings and transfer efficiencies

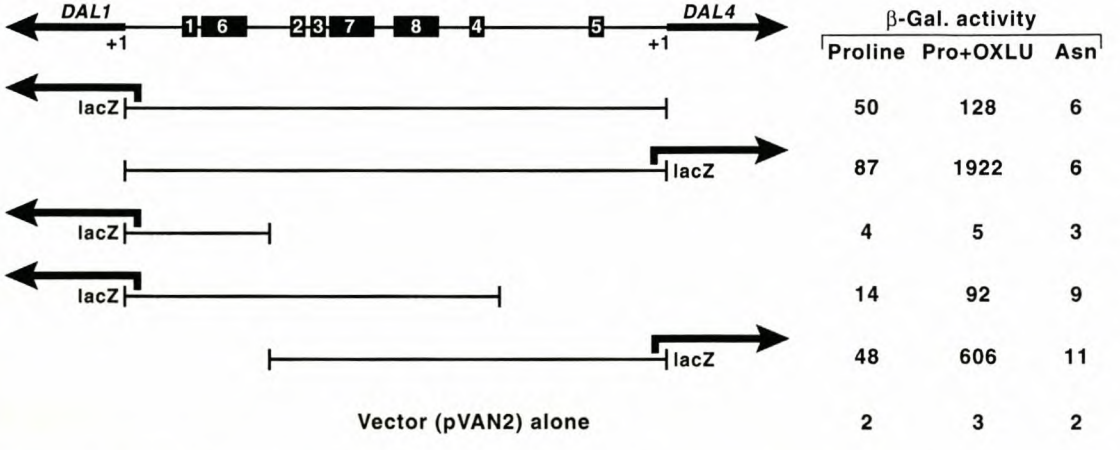

Figure 2. 5' Deletion analysis of the *DAL1* and *DAL4* upstream regions. A schematic of the *DAL1-DAL4* intergenic region (*top*) indicates UAS_{NTR} (*small black boxes, 1-5*) and UIS_{ALL} (*large boxes, 6-8*) homologous sequences. Coordinates indicate the 5' termini of the remaining promoter DNA. *Arrows marked lacZ* indicate the intergenic region fusion point *lacZ*. Transformants (TCY1 recipient) were grown in YNB proline (0.1%) medium without (*Proline*) and with (*Proline + OXLU*) 0.5 mM OXLU; 0.1% asparagine (*Asn*) was also used as a nitrogen source. β-Galactosidase (β-Gal.) activities are expressed in Miller Units

Contribution of UAS_{NTR}-homologous sequences to *DAL4* expression

To determine the contribution of individual intergenic GATA sequences to *DAL4* expression, each was destroyed by substitution mutations that did not otherwise alter the intergenic region. Transformants were assayed in both the presence and absence of inducer, because UAS_{NTR} and UIS_{ALL} are known to function synergistically in supporting inducer-mediated transcription (Yoo et al. 1989). The single mutant plasmids were also assayed in cells grown with asparagine as sole nitrogen source; and none of them supported reporter gene expression (data not shown). Activities observed in wild-type cells grown in inducer-free medium were too small in some cases to confidently compare alleles (Fig. 3). However, *DAL4* expression was highly inducible (fold induction) with all but two plasmids.

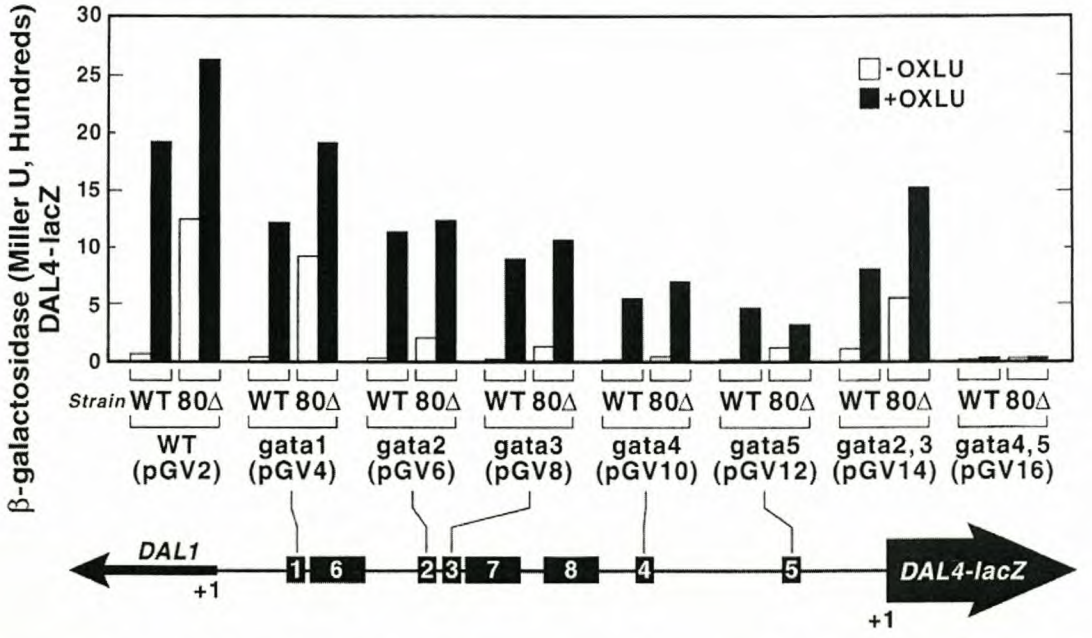

Figure 3. Single and combinational mutation analyses of putative *GATA* elements in the *DAL1-DAL4* intergenic region and their contribution to *DAL4-lacZ* expression. Elements were mutated in the context of the full-length intergenic region. *Small lettering* indicates the specific *GATA* element(s) mutated with the corresponding plasmid *in brackets below*. The native *DAL4-lacZ* fusion (*large arrow*) is indicated by *WT* (*pGV2*). These plasmids were transformed into strains TCY1 (*WT*) and TCY17 (*dal80Δ*). Transformants were grown in YNB-proline media in the absence (*open bars*) or presence (*solid bars*) of 0.5 mM OXLU

The fold-induction declined both with pGV14 (*gata2,3*), due to higher uninduced and lower induced levels, and with pGV16 (*gata4,5*), because expression was largely lost. Single *gata1* (pGV4), *gata2* (pGV6), and *gata3* (pGV8) mutations, while not significantly altering the fold of induction, decreased the induced β-galactosidase levels incrementally 1.6- to 3.4-fold; and *gata4* (pGV10) and *gata5* (pGV12) mutations resulted in somewhat greater decreases, 3.4 and 4.0-fold respectively (Fig. 3). The loss of induced *lacZ* expression in a *gata2,3* double mutant (pGV14) was roughly the same as seen in either single mutant, arguing that either sequence would suffice (Fig. 3). In contrast, a *gata4,5* double mutant (pGV16) was synthetic, reducing β-galactosidase production to background (Fig. 3). While each GATA sequence contributes to overall induced *DAL4* expression, the pair of GATAs closest to the *DAL4* TATA elements are by far most crucial.

Evaluating the contributions of particular GATA sequences to *DAL1* and/or *DAL4* expression is complicated by the fact that such sequences are potential binding sites not only for Gln3p and Gat1p, but also for the repressor Dal80p (Cunningham et al. 1996). When this complication is eliminated by performing the experiment in a *dal80* mutant, uninduced expression levels can be easily compared. All but the *gata1* mutation decreased uninduced level expression 6- to 25-fold arguing that GATAs 2-5 all functioned (Fig. 3). Somewhat surprisingly, the *gata2,3* double mutation caused a significantly smaller decrease in the absence of inducer than either of the corresponding single mutations (pGV14, pGV6, pGV8). In the presence of inducer, the *gata4* (pGV10) and *gata5* (pGV12) mutations possessed the strongest phenotypes; the *gata4,5* double mutation (pGV16) totally destroyed *lacZ* expression (Fig. 3).

Contribution of UIS_{ALL}-homologous sequences to *DAL4* expression

In addition to the five GATA sequences, the *DAL1-DAL4* intergenic region contains three UIS_{ALL}-homologous sequences, potential binding sites for the Dal82p that is required for inducer-dependent transcription. Mutating individual UIS_{ALL}-homologous sequences [*UIS6* (pGV22)and *UIS8* (pGV34)] reduced induced *DAL4-lacZ* expression 1.7- and 2.5-fold, respectively; and the *UIS7* mutation (pGV28) was without effect (Fig. 4). In contrast to expectation, uninduced *DAL4-lacZ* expression increased 6.5-fold in a *uis7* mutant relative to wild-type (Fig. 4). Double and triple *uis* mutations produced stronger phenotypes. The *uis6,7,8* triple mutation (pGV42) supported the least β-

galactosidase production, which was also inducer-independent (Fig. 4). Any double mutant containing a *uis8* mutation [*uis7,8* (pGV38) and *uis6,8* (pGV40)] supported a similarly low induced *lacZ* expression, which was less than in the *uis6,7* mutant (pGV36). Any double mutant containing a *uis7* mutation exhibited significantly more expression in the absence of inducer (Fig. 4).

While all three UIS_{ALL}-homologous sequences contributed to overall inducer-responsive *DAL4* expression, their contributions were not equal; and in one case, the element had the opposite function to the others. Inducer-responsiveness of the *DAL* genes depends upon Dal82p, which binds to UIS_{ALL} elements (Dorrington and Cooper 1993). Therefore, as a control, we compared results obtained in a wild type with those in *dal82*Δ. Expression in *dal82*Δ strain SS400 was unaffected by inducer (Fig. 4). However, in every case, the uninduced expression level was greater than that in the wild-type, further substantiating that one or more of the UIS_{ALL}–homologous sequences could behave as a negative regulator when inducer is absent.

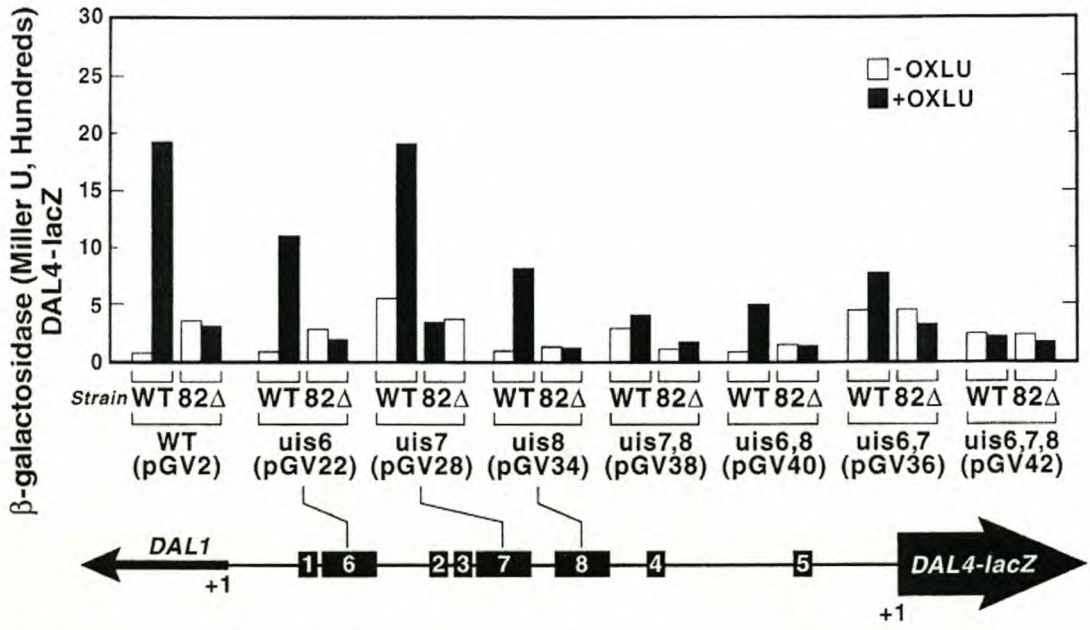

Figure 4. Single and combinational mutation analyses of putative UIS_{ALL} elements present in the *DAL1-DAL4* intergenic region and their contribution to *DAL4-lacZ* expression. UIS_{ALL}-homologous elements were mutated in the context of the full-length intergenic region. *Small lettering* indicates the specific UIS_{ALL} element(s) mutated with the corresponding plasmid *in brackets below*. The native *DAL4-lacZ* fusion is indicated by *WT* (*pGV2*). Plasmids were transformed into strain TCY1 (*WT*) or SS400 (*dal82*Δ). Transformants were grown in YNB-proline medium in the absence (*open bars*) or presence (*solid bars*) of 0.5 mM OXLU

Contribution of UAS_{NTR}-homologous sequences to *DAL1* expression

We similarly evaluated contributions of the five UAS_{NTR}-homologous sequences to *DAL1* expression. In contrast to *DAL4, DAL1* expression was much less inducible (less than 2.5-fold) and mutating the GATA sequence most proximal to *DAL1* (*gata1*; pGV3) had no significant effect on expression observed in a wild-type strain (Fig. 5). Mutating each of the remaining four GATAs decreased induced *DAL1-lacZ* expression less than 2-fold; and similar results occurred with *gata2,3* and *gata4,5* double mutant plasmids.

Stronger mutant phenotypes occurred when the analysis was performed in a *dal80* mutant background. The first and most striking characteristic of the data is seen by comparing *DAL4-lacZ* and *DAL1-lacZ* expression. For *DAL4-lacZ*, uninduced β-galactosidase production increases in *dal80* mutants, but never exceeds levels seen in an induced strain containing a wild-type plasmid (Fig. 3, pGV2). In contrast, uninduced *DAL1-lacZ* expression in a *dal80* strain background increases to a much higher level than induced *DAL1-lacZ* expression in both wild-type and *dal80* mutant strains (Fig. 5, pGV1). This profile is characteristic of a NCR-sensitive gene whose expression is largely inducer-independent, e.g., *DAL5* (Cooper 1996).

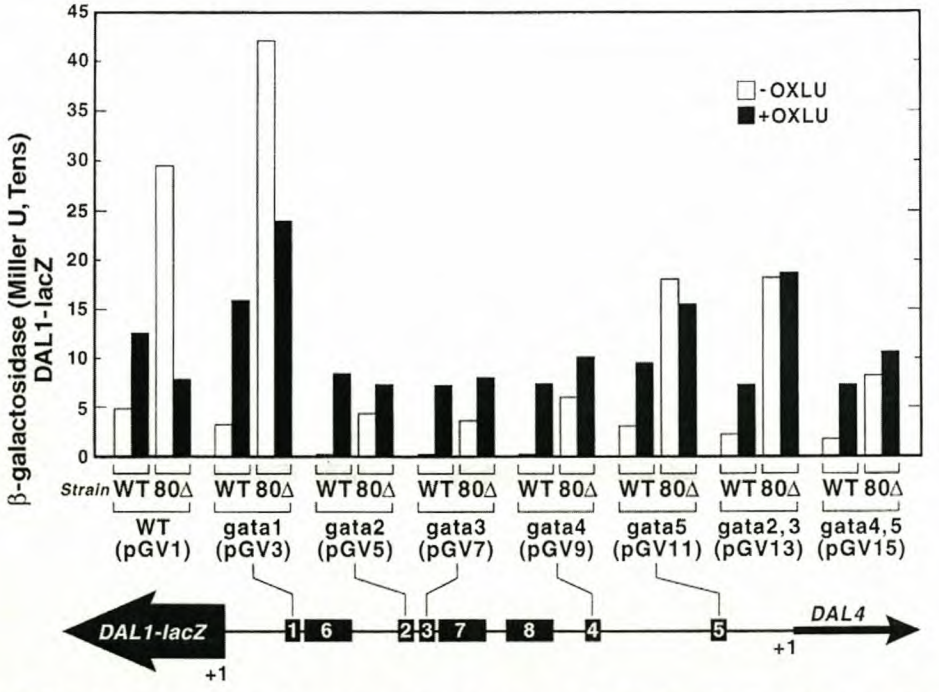

Figure 5. Single and combinational mutation analyses of putative *GATA* elements in the *DAL1-DAL4* intergenic region and their contribution to *DAL1-lacZ* expression. The experiment was performed as described in Fig. 3, except that *lacZ* was fused to the *DAL1* end of the intergenic region (*large arrow*)

In the *dal80* mutant background, the differing contributions of the GATA-homologous sequences to overall *DAL1* expression are more apparent. Mutation of *GATA2*, *3*, or *4* decreased uninduced expression 4.8- to 8.0-fold relative to wild type (Fig. 5, pGV5, pGV7, pGV9). However, *lacZ* expression in a *gata5* mutant (pGV11) decreases less than 2-fold relative to wild-type (pGV1), arguing that its contribution to *DAL1* expression is quite limited. In contrast, mutating the *GATA1* sequence (pGV3) increases the amount of *lacZ* expression observed in a *dal80* mutant significantly above the level supported by wild-type pGV1.

Contribution of UIS_{ALL}-homologous sequences to *DAL1* expression

Induced *β*-galactosidase production, from wild-type *DAL1-lacZ* pGV1, was about 2.5-fold greater than the uninduced level (Fig. 6). The only effect of mutating the UIS most proximal to *DAL1* (*UIS6*) was a 3.0-fold decrease in uninduced reporter expression (Fig. 6, pGV21). Mutating the UIS most proximal to *DAL4* (*UIS8*) decreased induced *β*–galactosidase production from *DAL1-lacZ*, but only very modestly; and it did not affect the uninduced levels (Fig. 6, pGV33). The *uis6,8* double mutant (pGV39) exhibited uninduced expression similar to that supported by the *uis6* mutation and an induced level more similar to that seen with the *uis8* mutation (Fig. 6).

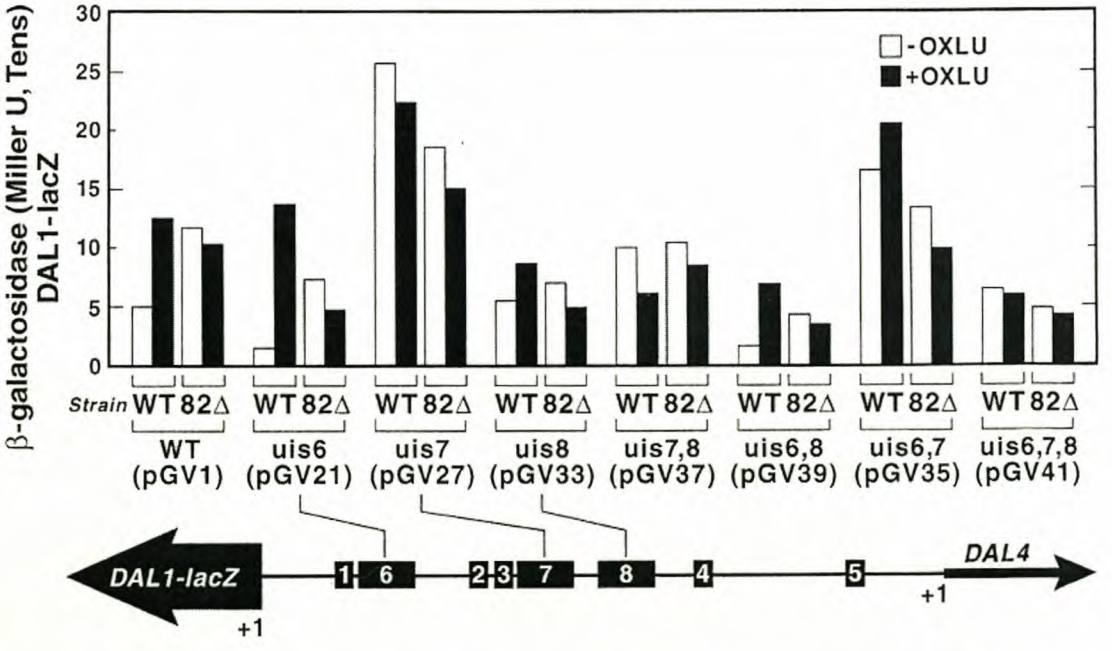

Figure 6. Single and combinational mutation analyses of putative UIS_{ALL} elements in the *DAL1-DAL4* intergenic region and their contribution to *DAL1-lacZ* expression. The experiment was performed as described in Fig. 4, except that *lacZ* was fused to the *DAL1* end of the intergenic

region (*large arrow*)

Mutating the central UIS_{ALL}-homologous sequence, *UIS7*, generated the strongest and also most surprising phenotype (Fig. 6, pGV27). Uninduced and induced *lacZ* expression increased 5.0- and 2.0-fold, respectively. In other words, the *UIS7* sequence behaved more as a negative than a positive regulator of *DAL1* expression. An analogous response has been reported for an inducer-responsive element situated upstream of *CAR1* (Kovari et al. 1990). The *uis6,7* double mutant exhibited a phenotype similar to that of a *uis7* single mutant, with respect to wild-type. In contrast, a *uis7,8* double mutant exhibited a phenotype that more closely resembled the *uis8* single-mutant phenotype. Together, these data suggest the *UIS7* sequence down-regulates *DAL1* expression, supported in part by *UIS8*. It must be emphasized, however, that the negative regulation is stronger than the positive. When the experiment was repeated in a *dal82*Δ, the major effect observed was a loss of inducer responsiveness, i.e., all of the uninduced *lacZ* levels were as high or higher than the induced levels (Fig. 6).

Discussion

The above data assess the contributions to *DAL1*- and *DAL4-lacZ* expression of each UAS_{NTR}- or UIS_{ALL}-homologous sequence in the *DAL1-DAL4* intergenic region. In a wild-type background, in the presence of inducer, all five GATAs appear to participate in *DAL4-lacZ* expression with *GATA2-GATA3* and *GATA4-GATA5* making relatively equal contributions. The *gata2,3* and *gata4,5* double mutants, however, argue that *GATA4* and *GATA5* are the most important. Similar conclusions are drawn from the experiments in a *dal80* background grown with inducer. Without inducer, however, only GATAs2-5 appear to function.

Single mutation data argue that induced *DAL4* expression depends somewhat more heavily on *UIS8* than *UIS6*, while *UIS7* is not a positive participant in *DAL4* induction. Data with double and triple mutations, however, suggest that *UIS7* can play a limited positive role if *UIS8* and *UIS6* are mutated (Fig. 4, pGV40, pGV42). In contrast with expectation, *UIS7* appears to play a negative role in the absence of inducer.

From previous detailed studies of the *CAR1*, *CAR2*, *DAL5*, *DAL7*, and *DUR1,2* promoters, one would have predicted that *GATA4*, *GATA5*, and *UIS8* were the most likely participants in *DAL4-lacZ* expression, based both on their sequences and on their

orientation and location (G. Genbauffe, H. El Berry, J.R. Daugherty, and T.G. Cooper, unpublished data; Kovari et al. 1990; Park et al. 1999; Rai et al. 1989, 1999). Here data and expectation are similar, but not congruent. However, previous literature also report that a DNA fragment carrying *UIS7* is a much better competitor of a standard Dal82p-binding DNA fragment than *UIS8* (Dorrington and Cooper 1993). From this, *UIS7* would have been hypothesized to be the more likely candidate as the *cis*-acting element mediating induced *DAL4* expression. Data presented here are consistent with both *UIS7* and *UIS8* participating in gene function but in opposite ways which is not predictable a priori.

For *DAL1-lacZ* expression, predictions of the participating *cis*-acting elements are less easily made, but the most reasonable choices are *GATA1*, *GATA2*, and *GATA3*. UIS elements cannot be considered significant because *DAL1* expression is so little affected by addition of inducer. From data with single intergenic mutations in wild-type background, *GATA2-4* are about equal participants in the absence of inducer; the participation of *GATA5* is marginal. However, from double mutants, uninduced *DAL1-lacZ* expression can proceed quite well in the absence of both *GATA2* and *GATA3*. Mutation of *GATA1* yields a slight increase in the induced level of expression, whereas in *gata2, gata3,* and *gata4* mutants, the induced expression is slightly depressed. Although most of the conclusions drawn from the wild-type are substantiated by data in the *dal80* background, there is a marked change. The wild type plasmid (pGV1) exhibited greater expression in the absence of inducer than in its presence; and *GATA1* is responsible for this pattern of expression.

Expectation and observation differ even more for the case of UIS participation in *DAL1* expression. First and foremost, since *DAL1* expression is not particularly inducer responsive (Fig. 1), UIS_{ALL} participation is not expected. However, detailed analysis reveals that the UIS elements definitely participate in *DAL1* transcription. Second, to the extent that *DAL1* is OXLU-responsive, one would predict *UIS6* and *UIS7* to most likely account for any induction observed. *UIS6*, the UIS_{ALL}–homologous sequence most proximal to *DAL1*, does not appear to participate; and, surprisingly, *UIS7* appears to be a negative rather than positive regulator. The putative element least expected to participate in *DAL1* expression, *UIS8,* is the most necessary. Considering single and double mutant data together, *UIS8* continues to be most necessary.

The appearance of *UIS7* as a putative, negatively acting element deserves further comment as, at face value, it seems to contradict much of the literature concerning UIS_{ALL} and Dal82p. A similar phenomenon was observed during dissection of the *CAR1* promoter (Kovari et al. 1990). In that instance, placing an arginine-dependent UAS element downstream of a constitutively acting UAS, UAS_{C2}, resulted in a six-fold decrease in reporter gene expression when glutamate was provided as sole nitrogen source. This "negative regulation" disappeared, however, when arginine was used in place of glutamate as the nitrogen source (Fig. 7, pLK78, pLK105 in Kovari et al. 1990). The explanation offered for *CAR1* was that if a protein that is not functioning in transcription, i.e., serving as a UAS due to the absence of the inducer (arginine) upon which its operation depends, binds to a site downstream of a functioning UAS (UAS_{C2}), it will repress transcription supported by it. We suggest that *UIS7* is behaving similarly. *UIS7* is not functioning as a UIS element as far as *DAL1* expression is concerned and hence behaves like a negatively acting element, because it is situated between the *cis*-acting elements responsible for *DAL1* transcription and the TATA element.

This work focused only on known allantoin-pathway, *cis*-acting elements. The *DAL1-DAL4* intergenic region may well contain other *cis*-acting elements that have gone unnoticed, but are important to the operation of the elements we analyzed. That such additional elements also participate in expression of allantoin and arginine pathway genes is well documented (Dubois and Messenguy 1997; Park et al. 1999; Rai et al. 1999; Smart et al. 1996). The presence of such elements would only complicate a straightforward correlative analysis yet further. An example of this may be seen with respect to the *DAL1* and *DAL4* TATA elements. *DAL4* is expressed much more strongly than *DAL1*. This correlates with the presence of three potentially strong TATA sequences, TATAAA, TATATA, and TATAT immediately upstream of *DAL4*, whereas only two such sequences, TATAG and TATAT, appear upstream of *DAL1*.

Beyond identifying the relative contributions of the *DAL1-DAL4* *cis*-acting sequences, these data demonstrate that:

1. The actual participation of various UAS_{NTR}- and UIS_{ALL}-homologous sequences in *DAL1*- and *DAL4-lacZ* expression could be predicted with quite limited success, even though they were based on previously reported, detailed analyses of five highly related promoters.

2. *DAL1* and *DAL4* do not equally share a set of *cis*-acting elements equally; and, further, elements for the expression of the two genes cannot be predicted from their locations.
3. Even shared elements do not always function in the same way for the two genes.
4. The potential participation of a given element and its contribution to *DAL1* and *DAL4* expression is not only a function of the element and its location, but also of the other elements are functioning in proximity to it.
5. The UIS_{ALL}–homologous sequence predicted, on the basis of in vitro DNA-binding experiments (Dorrington and Cooper 1993), as being the most likely to be responsible for induced *DAL4* expression did not contribute to induction.

The *DAL1* expression profile also demonstrates that the presence of both UAS_{NTR}- and UIS_{ALL}-homologous sequences in a gene's promoter region is not necessarily indicative of inducibility as expected a priori. Therefore, correlations of genome-wide expression profiles with the presence of sequences homologous to known transcription factor-binding sites or found repeated within the promoter sequences of co-regulated genes may be a legitimate starting point of investigating their potential function. However, with our current state of technology, they cannot be considered as an end-point with which to draw rigorous conclusions.

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CHAPTER 4

Ammonia Regulates *VID30* Expression and Vid30p Function Shifts Nitrogen Metabolism towards Glutamate Formation Especially when *Saccharomyces cerevisiae* is Grown in Low Concentrations of Ammonia

G.K. van der Merwe, T.G. Cooper, and H.J.J. van Vuuren

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Ammonia Regulates *VID30* Expression and Vid30p Function Shifts Nitrogen Metabolism towards Glutamate Formation Especially when *Saccharomyces cerevisiae* is Grown in Low Concentrations of Ammonia

G.K. van der Merwe[1], T.G. Cooper[2], and H.J.J. van Vuuren[1]

[1]Wine Research Center, Faculty of Agricultural Sciences, University of British Columbia, Vancouver, BC V6T 1Z4, Canada and the [2]Department of Microbiology and Immunology, University of Tennessee, Memphis, TN 38163, U.S.A.

The GATA-family proteins Gln3p and Gat1p mediate nitrogen catabolite repression (NCR)-sensitive transcription in *Saccharomyces cerevisiae.* When cells are cultured with a good nitrogen source (glutamine, ammonia), Gln3p and Gat1p are restricted to the cytoplasm, whereas with a poor nitrogen source (proline), they localize to the nucleus, bind to the GATA sequences of NCR-sensitive gene promoters, and activate transcription. The target of rapamycin-signaling cascade and Ure2p participate in regulating the cellular localization of Gln3p and Gat1p. Rapamycin, a Tor protein inhibitor, like growth with a poor nitrogen source, promotes nuclear localization of Gln3p and Gat1p. *gln3*Δ and *ure2*Δ mutants are partially resistant and hyper-sensitive to growth inhibition by rapamycin, respectively. We show that a *vid30*Δ is more rapamycin-sensitive than wild type but less so than a *ure2*Δ. *VID30* expression is modestly NCR-sensitive, responsive to deletion of *URE2*, and greatly increases in low ammonia medium. Patterns of gene expression in a *vid30*Δ suggest that the Vid30p function shifts the balance of nitrogen metabolism towards the production of glutamate especially when cells are grown in low ammonia. *CAN1*, *DAL4*, *DAL5*, *MEP2*, *DAL1*, *DAL80*, and *GDH3* transcription is down-regulated by Vid30p function with proline as nitrogen source. An effect, however, that could easily be indirect.

Saccharomyces cerevisiae has evolved to live in widely varying nutritional environments from rich YPD medium in a laboratory flask to poor, depleted soil. A significant contributor to the organism's success is its ability to selectively use a variety of nitrogen sources. The basis of this selectivity is nitrogen catabolite repression

(NCR)[1]. Expression of nearly all genes whose products participate in the uptake and catabolism of nitrogenous compounds are NCR-sensitive and dependent upon two GATA-family transcription factors, Gln3p and Gat1p/Nil1p (see Refs. 1-4 for reviews of the field). When nitrogen availability limits growth, as occurs with poor nitrogen sources such as proline, Gln3p and/or Gat1p bind to the GATA sequences upstream of NCR-sensitive genes and activate their transcription (5-11). However, in nitrogen excess, the GATA sites are unoccupied because Gln3p and Gat1p are excluded from the nucleus (12-15). Ure2p, which binds to Gln3p and Gat1p (15-17), has long been known to be a negative regulator of NCR-sensitive gene expression and more recently Gln3p- and Gat1p-mediated transcription (12-20).

In a manner that appears to be similar to that which occurs when cells are provided with a poor nitrogen source, the macrolide antibiotic rapamycin participates in the regulation of Gln3p and Gat1p intracellular localization (12,15,17). Rapamycin specifically binds Fpr1p (peptidylprolyl isomerase), and the resulting complex binds to and inactivates Tor1p and Tor2p protein kinases (21,22). Inactivation of Tor1p/Tor2p has been implicated in a broad range of cellular functions including cell cycle progression (22-24), translation initiation (24), ribosome biosynthesis (13), autophagy (25,26), amino acid permease stability (27), and microtubule assembly (28). More recently, genome-wide transcriptional analyses have shown that a substantial fraction of the total transcriptome changes following rapamycin addition to the medium (13,14, 29).

A group of genes significantly affected in the transcriptome analyses were those whose expression is NCR-sensitive, i.e., Gln3p-/Gat1p-dependent (13,14, 29). Deletion of *GLN3* results in partial resistance to rapamycin, while *ure2*Δ mutants are hypersensitive (13). The phosphorylation state of Gln3p/Gat1p and Ure2p correlates with these data. Gln3p, Gat1p, and Ure2p are hyperphosphorylated in cells provided with excess nitrogen and underphosphorylated when nitrogen is limiting or rapamycin is added to the medium (12-14,17). Together these data suggest that the Tor proteins play a role in transduction of the signal that responds to the cell's nitrogen supply.

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[1] The abbreviation used is: NCR, nitrogen catabolite repression.

A search of the literature for rapamycin-related molecules identified a protein about which little is known, the product of the YGL227w ORF which has been given the temporary designations Vid30p and Tin1p[2]. Since so little is known about this gene, we investigated its transcription profile and mutant phenotype. Here we show that *VID30* expression possesses characteristics expected of an NCR-sensitive gene. Although a *vid30*Δ mutant exhibits alterations in NCR-sensitive gene expression, the data we collected are most consistent with the suggestion that Vid30p function shifts nitrogen metabolism towards the formation of glutamate, especially when cells are grown in limiting ammonia.

EXPERIMENTAL PROCEDURES

Strains and Media – S. cerevisiae strains used in this work are all derivatives of wild type BY4742 (Table I). Yeast were cultured at 30°C in YNB-glucose medium (0.17% Yeast Nitrogen Base without ammonium sulphate or amino acids (Difco), 2% glucose; quantities of amino acids (in micrograms) were provided to cover auxotrophies where necessary). Proline (0.1%), ammonium sulphate (0.05 or 7.6 mM [0.1%]), or glutamine (0.1%) were provided as sole nitrogen sources. For all experiments, cells were harvested at a density of $A_{600nm} \sim 0.8$ and used for total RNA extraction.

The transcriptional effects of overexpressing *VID30* were analyzed by growing transformants (pYES2 or pYES-V) of BY4742 in YNB-raffinose/galactose medium (1.5% raffinose + 0.5% galactose) with proline (0.1%) or glutamine (0.1%). Effects of *VID30* overexpression on cell growth were determined by growing wild type transformants (pYES2 or pYES-V) to exponential phase at 30°C in YNB-glucose (2%) – ammonium sulfate (0.1%) medium. Cells were harvested, washed, resuspended in sterile water, and streaked onto YNB plates containing either 2% glucose or 2% galactose and 0.1% proline, 0.1% glutamine, or 0.1% ammonium sulphate as sole carbon and nitrogen sources, respectively.

To analyze the effects of rapamycin, *vid30*Δ, *ure2*Δ, *gln3*Δ, and wild type strains growing exponentially in YPD medium were harvested, washed, resuspended in sterile

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[2] J.M. Cherry, C. Ball, K. Dolinski, S. Dwight, M. Harris, J.C. Matese, G. Sherlock, G. Binkley, H. Jin, S. Weng, and D. Botstein, Saccharomyces Genome Data Base (genome-www.stanford.edu/Saccharomyces/cgi-bin/SGD/locus.pl?locus=VID30).

water, and streaked onto YPD or YPD + 50 ng/ml rapamycin solid media. The plates were incubated at 30°C, 3 days for YPD and 5 days for YPD + rapamycin.

TABLE I.
Strains used in this research

Strain	Genotype
S. cerevisiae	
BY4742	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*
BY14594	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 vid30Δ*
BY11983	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 ure2Δ*
BY10173	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 gln3Δ*
E. coli	
DH5α	*E. coli* DH5α F'/*endA1 hsdR17*($r_K^- m_K^+$) *supE44 thi1 recA1 gyrA* (Nal^r) *relA1* Δ(*lacZYA-argF*)$_{U169}$ (*m80lacZΔM15*)

Northern Analysis - Total RNA was isolated as described previously (31). Poly(A)$^+$ RNA was obtained using mRNA Isolation Kits (Roche Molecular Biochemicals) according to the manufacturer's recommendations. Samples of poly(A)$^+$ RNA were resolved on 1.4% agarose-formaldehyde gels and transferred to either GeneScreen Plus nylon membranes 66 (PerkinElmer Life Sciences) or positively charged nylon membranes (Roche Molecular Biochemicals). Double-stranded DNA probes (radioactively labeled using a Random Primed DNA Labeling Kit [Roche Molecular Biochemicals]) were synthesized by PCR using the oligonucleotides in Table II as primers. pC4 (loading and transfer standard) and pTSC317 (*DAL80*) were also used as probes (32,33). Oligonucleotides used as probes for *GDH1* and *GDH3* (Table II) were end-labeled using polynucleotide kinase (Roche Molecular Biochemicals). Standard prehybridization, hybridization and washing conditions were followed (31).

Plasmid Construction – Overexpression of *VID30* was achieved by fusing it to the *GAL1* promoter in multicopy pYES2 (Invitrogen Corporation, Carlsbad, CA). *VID30* was amplified from *S. cerevisiae* genomic DNA using heat-stable DNA polymerase PWO, and primers VKpn-5 and VXho-3 (Table II), into which *Kpn*I and *Xho*I sites had been engineered, respectively. The resulting PCR product was digested with *Kpn*I and *Xho*I, yielding a 3,028 base pair fragment, which was cloned into the *Kpn*I and *Xho*I sites of pYES2 to create pYES-V.

TABLE II.
Oligonucleotides used for PCR amplifications

Target gene	Oligonucleotide
CAN1	CAN15: 5'-ATGAGCCGGTCACAACCCTC-3'
	CAN13: 5'-GATGGAAGCGACCCAGAACT-3'
DAL1	DAL15: 5'-TGGCGTTCGCGGGTTCAAAG-3'
	DAL13: 5'-TGCAACTTTAGTTTAACGTC-3'
DAL4	DAL45: 5'-ATGGCTAACGACGCTCT-3'
	DAL43: 5'-TATGACACAATAGATGT-3'
DAL5	DAL55: 5'-CAGTATTCATGGGTTACTTCC-3'
	DAL53: 5'-TAAGGTTCTCCAGCCTTTAAT-3'
GDH1[a]	GDH1-P:5'-ACCTTACCGTCCTTAGTGTACTTCTTGGCATAGTCG-3'
GDH2	GDH25: 5'-ATAACAAAAATCGCGGTG-3'
	GDH23: 5'-TATCCTTACCATGCTCCA-3'
GDH3[a]	GDH3-P:5'-GTGTTTGTATTTTTTTCCGTAGAGTACTCTTGTGCGGCCTGT-3'
GLN1	GLN15: 5'-ATGGCTGAAGCAAGCATC-3'
	GLN13: 5'-CTTACCGGCACCAACACC-3'
GLT1	GLT15: 5'-TATTAGGTTGGAGAAACG-3'
	GLT13: 5'-AGTTCTAAAACGTTATCC-3'
HHF1	H45: 5'-GGCCGGATCCATGTCCGGTAGAGGTAAAGG-3'
	H43: 5'-GCCGAATTCTTAACCACCGAAACCGTATAAGG-3'
MEP2	MEP25: 5'-GTCTTACAATTTTACAGG-3'
	MEP23: 5'-TACCCAATTTGACCAACC-3'
VID30	VID305: 5'-ATGTCTGAATATATGGATGA-3'
	VID303: 5'-TCACGTTCGGATAAAACGGG-3'
	VKpn-5: 5'-AATTGGTACCAAATGTCTGAATATATGGAT-3'
	VXho-3: 5'-TTGGCTCGAGAATGACTGATATCACATGGC-3'

[a] Oligonucleotides were end-labeled and used as probes

RESULTS

VID30 Expression Is NCR-sensitive and Rapamycin responsive – To ascertain whether Vid30p played a part in the rapamycin cascade that participates in the regulation of NCR-sensitive gene expression, we compared the effects of the TOR protein inhibitor, rapamycin, on wild type and mutant cells. Growth of *ure2*Δ strains are hypersensitive to rapamycin, while *gln3*Δ mutants are more resistant than wild type (13,17). A *vid30*Δ mutant is slightly less hypersensitive than a *ure2*Δ strain (Fig. 1),

implying a relationship potentially exits between the TOR signaling cascade and Vid30p function. This prompted us to determine whether *VID30* expression was NCR-sensitive by assaying steady-state *VID30* mRNA derived from cells cultured in YNB-proline (nitrogen-derepressive) and YNB-glutamine (nitrogen-repressive) media. *VID30* expression is modestly NCR-sensitive (Fig. 2*A*); this result can be even more clearly observed when the autoradiogram is highly overexposed. The NCR-sensitivity of *VID30* expression and rapamycin-sensitivity of a *vid30*Δ mutant predicted a response of *VID30* expression to deletion of *URE2* or addition of rapamycin to the medium. In agreement with these expectations, *VID30* expression increases following addition of 200 ng/ml rapamycin to nitrogen rich medium, but less dramatically so than occurs with the control gene, *DAL5* (Fig. 2*B*). *VID30* expression in cells provided with high ammonia or glutamine (excess nitrogen) also increases when *URE2* is deleted (Fig. 2*C*). It is significant that the magnitude of NCR-sensitivity, rapamycin-mediated induction, and *ure2*Δ-generated derepression of *VID30* expression are quantitatively similar. Together, these data argue that *VID30* expression is modestly NCR-sensitive.

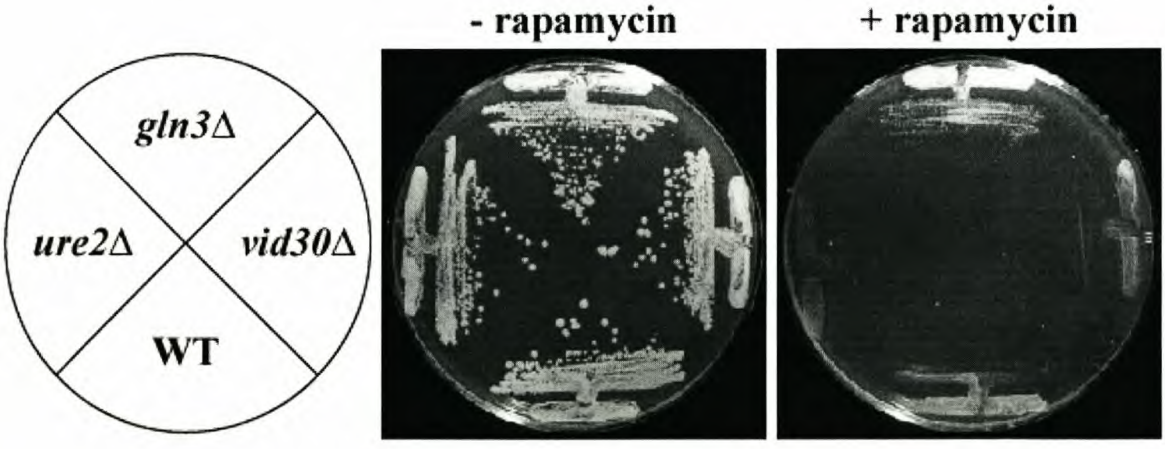

FIG. 1. **Deletion of *VID30* confers hypersensitivity to rapamycin.** Wild type (*WT*; BY4742), *gln3*Δ (BY10173), *ure2*Δ (BY11983), and *vid30*Δ (BY14594) strains grown on rich medium containing (+ *Rap*) or devoid (- *Rap*) of rapamycin (50 ng/ml). Plates were incubated at 30°C for 3 (-*Rap*) and 5 days (+*Rap*), respectively.

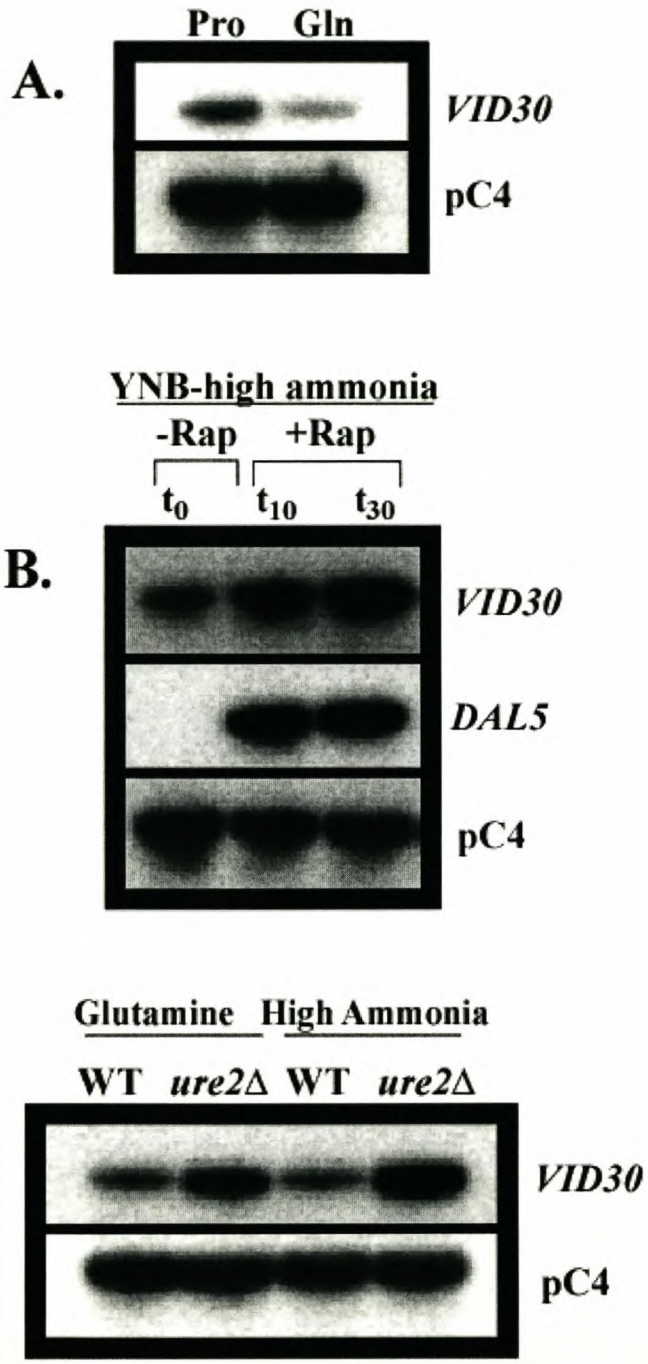

FIG. 2. ***VID30* expression is NCR-sensitive and induced by the TOR protein inhibitor rapamycin.** *A*, Northern blot analysis of poly(A)$^+$ RNA (1 μg/lane) from BY4742 grown in YNB-0.1% proline (*Pro*) and YNB-0.1% glutamine (*Gln*). *B*, Northern blot analysis of poly(A)$^+$ RNA (5 μg/lane) from BY4742 was grown in YNB-0.1% ammonium sulphate to late log phase (t_0), and then 10 minutes (t_{10}) and 30 minutes after (t_{30}) addition of rapamycin to a final concentration of 200 ng/ml to the growth medium. *C*, poly(A)$^+$ RNA (2.5 μg/lane) was extracted from strains BY4742 (*WT*) and BY11983 (*ure2*Δ) grown in either YNB-0.1% glutamine or YNB-0.1% ammonium sulphate. pC4 was used as the loading standard.

Vid30p Negatively Regulates Expression of Multiple Genes Associated with Nitrogen Catabolism – The similar hypersensitivities of *vid30*Δ and *ure2*Δ mutants to rapamycin prompted us to investigate whether Vid30p, like Ure2p, might be a negative regulator of NCR-sensitive gene expression. We analyzed expression of representative NCR-sensitive genes in wild type and *vid30*Δ strains. Included in the analysis were genes encoding (i) permeases: *MEP2* (low capacity ammonia), *DAL4* (allantoin), *DAL5* (allantoate), and *CAN1* (basic amino acid); (ii) enzymes for the interconversion of ammonia, glutamate, and glutamine: *GDH1* (NADP-glutamate dehydrogenase), *GDH3* (NADP-glutamate dehydrogenase), *GDH2* (NAD-glutamate dehydrogenase), *GLN1* (glutamine synthetase), and *GLT1* (glutamate synthase (GOGAT, glutamine amide: 2-oxoglutarate aminotransferase)); (iii) an enzyme participating in catabolism of allantoin: *DAL1* (allantoinase); and (iv) the GATA-specific transcriptional repressor: *DAL80*.

Deletion of *VID30* increases *CAN1*, *DAL4*, *DAL5*, *MEP2*, *DAL1*, *GDH3*, and *DAL80* mRNA levels 2-10-fold in YNB-proline-cultured cells (Fig. 3*A*). However, the NCR-sensitivity of these genes is not affected, *i.e.* there is no alteration of expression when a *vid30*Δ mutant is growing in glucose-glutamine medium (Fig. 3*A*). At this point, we cannot rigorously distinguish whether the effects observed when a *vid30*Δ mutant is grown in glucose-proline medium are direct or indirect.

In contrast to typical NCR-sensitive genes, the expression profiles of genes associated with the interconversion of ammonia, glutamate, and glutamine (*GDH1*, *GDH2*, *GLN1* and *GLT1*) are quite different in two respects (Fig. 3*B*): (i) these genes are expressed more or less equivalently when wild type and *vid30*Δ strains are grown in YNB-proline medium, and (ii) their expression is markedly increased when a *vid30*Δ mutant growing in YNB-glutamine medium is compared to wild type; we estimate the increase to be in the range of 2-10-fold (Fig. 3*B*). These results argue that Vid30p negatively regulates *CAN1*, *DAL4*, *DAL5*, *MEP2*, *DAL1*, *GDH3*, and *DAL80* when cells are growing with proline as nitrogen source and *GDH1*, *GDH2*, *GLN1*, and *GLT1* when cells are provided with glutamine. However, deletion of *VID30* does not restore expression of the second group of genes in glucose-glutamine grown cells to the level seen in glucose-proline medium. They are more highly expressed in proline medium

regardless of whether or not Vid30p is present (Fig. 3*B*), indicating that negative regulation by Vid30p is shared, in a formal sense, by at least one other protein.

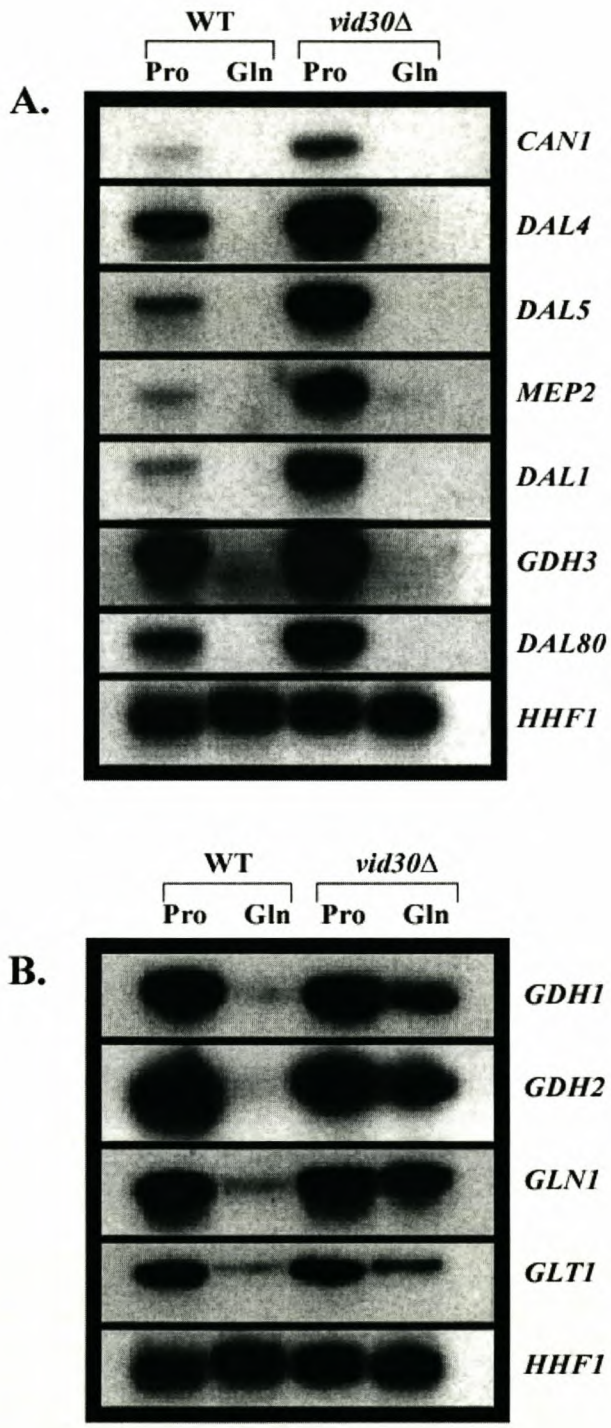

FIG. 3. **Expression of genes associated with nitrogen metabolism in wild type and *vid30*Δ strains provided with a repressive (glutamine) or derepressive (proline) nitrogen source.** Northern blot analysis of poly(A)$^+$ RNA (1 μg/lane), wild type (BY4742), and *vid30*Δ (BY14594) strains grown in either YNB-0.1% proline (*Pro*) or YNB-0.1% glutamine (*Gln*). Probes are indicated on the sides of the panels.

Low Ammonia-mediated VID30 Expression - Although *VID30* expression is modestly NCR-sensitive, more striking is the dramatic increase in *VID30* expression that occurs when cells are grown in medium containing low ammonia (0.05 mM) (Fig. 4*A*). *VID30* expression in low ammonia medium is more than 10-fold greater than with proline as nitrogen source (Fig. 4*A*). This high level of *VID30* expression is lost within 15 minutes after the ammonia concentration in the culture medium is increased to 0.1% (Fig. 4*B*).

The *VID30* expression we observe in low ammonia medium may conceivably be derived in two ways: (i) a response of *VID30* expression to the low concentration of ammonia or (ii) nitrogen starvation *per se*. To distinguish these possibilities, we divided a culture into two portions, one being transferred to low ammonia and the other a similarly low concentration of proline. As shown in Fig. 4*C*, the response was observed only in low ammonia medium, arguing against starvation as the driving force behind increased *VID30* expression.

Vid30p Acts as a Positive Regulator in Low Ammonia – High level *VID30* expression in low ammonia (0.05 mM) medium, and its loss when ammonia concentrations are high (7.6 mM (0.1 %)), prompted us to investigate Vid30p regulation of other nitrogen metabolism-related genes under similar experimental conditions. We analyzed expression of the two groups of genes described in Fig. 3 in wild type and *vid30*Δ strains. For *CAN1*, *DAL4*, *DAL5*, *MEP2*, and *DAL1*, the fold increase observed when comparing expression in low *versus* high ammonia-grown cells, is smaller in the *vid30*Δ mutant than the wild type (Fig. 5*A*). This result derives from the fact that deletion of *VID30* decreases expression of these genes in low ammonia medium with no demonstrable effect in high ammonia medium. These results suggest that Vid30p acts as a positive regulator of *CAN1*, *DAL4*, *DAL5*, *MEP2*, and *DAL1* gene expression in low ammonia. Taken together, these data and those in Fig. 3 suggest that Vid30p functions as a negative regulator with proline as nitrogen source and as a positive regulator with low ammonia.

With exception of *GDH2*, deletion of *VID30* does not effect the expression of ammonia-glutamate-glutamine interconversion genes when cells are growing in high

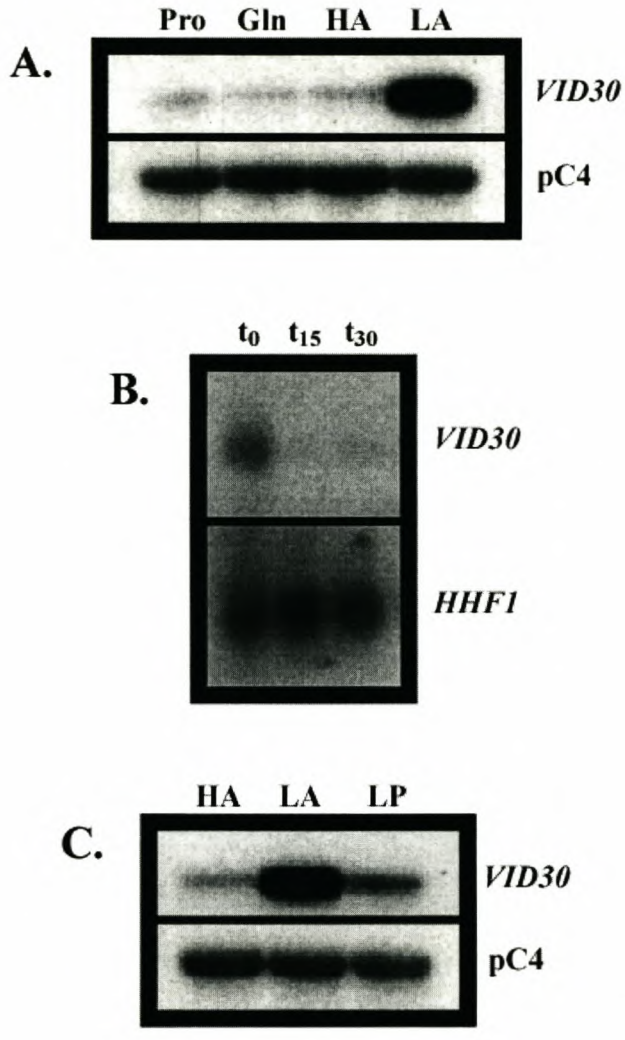

FIG. 4. ***VID30* expression is enhanced by low ammonia.** *A*, Northern blot analysis of poly(A)$^+$ RNA (1 μg/lane) from BY4742 grown in YNB-0.1% proline (*Pro*), YNB-0.1% glutamine (*Gln*), YNB-0.1% ammonium sulphate (*HA*), or YNB-0.05 mM ammonium sulphate (*LA*). *B*, BY4742 was grown in YNB-0.05 mM ammonium sulphate. Total RNA (30 μg/lane) were extracted from BY4742 cells grown to late log phase in YNB-0.05 mM ammonium sulphate (t_0), and then 15 minutes after (t_{15}) and 30 minutes after (t_{30}) addition of ammonium sulphate to a final concentration of 0.1% to the growth medium. *C*, Northern blot analysis of poly(A)$^+$ RNA (1 μg/lane) from BY4742 grown in YNB-0.1% ammonium sulphate (*HA*), YNB-0.05 mM ammonium sulphate (*LA*), and YNB-0.1 mM proline (*LP*). *HHF1* and pC4 were used as loading standards.

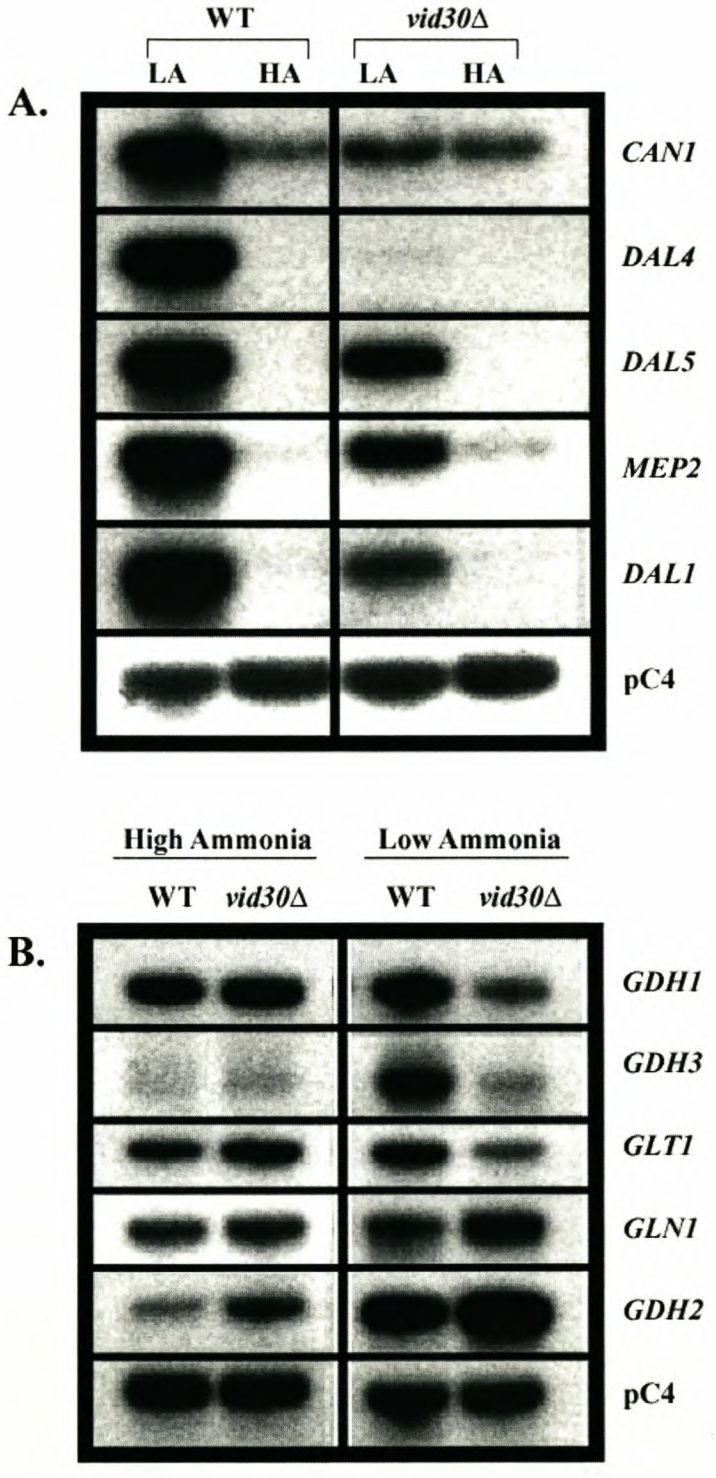

FIG. 5. **Expression of genes associated with nitrogen metabolism in wild type and *vid30*Δ strains grown in high and low ammonia.** Northern blot analysis of poly(A)$^+$ RNA (1 μg/lane) from wild type (BY4742) and *vid30*Δ (BY14594) strains grown in YNB-0.1% ammonium sulphate (HA) or YNB-0.05 mM ammonium sulphate (LA).

ammonia medium (Fig. 5*B*). The steady-state level of *GDH2* mRNA modestly increases in the *vid30*Δ. In addition, there is no detectable expression of *GDH3*, which is not surprising given its high NCR sensitivity and the fact that high ammonia is a repressive nitrogen source. On the other hand, in low ammonia medium, there is less expression of the *GDH1*, *GDH3*, and *GLT1* genes in *vid30*Δ than wild type. *GDH2* and maybe *GLN1* are the exceptions, *i.e.* their expression increases rather than decreases when the *vid30*Δ mutant is grown in low ammonia (Fig. 5*B*). Therefore, Vid30p behaves as a positive regulator for all of the interconversion genes in low ammonia-grown cells with exception of *GDH2* and *GLN1*, where control is modestly negative.

Overexpression of VID30 Inhibits Cell Growth and Alters Nitrogen-regulated Gene Expression - The NCR-sensitive, rapamycin-responsive expression of *VID30* prompted us to examine the effect of *VID30* overexpression on cell growth in different nitrogen conditions. *VID30* was fused to the *GAL1* promoter in pYES2, allowing *VID30* expression to be induced independently of the nitrogen source in the medium. Wild type strain BY4742, transformed with vector pYES2, grows similarly on YNB-glucose or YNB-galactose medium containing 0.1% ammonium sulphate or glutamine as sole nitrogen source (Fig. 6). The transformants grow more slowly, however, on

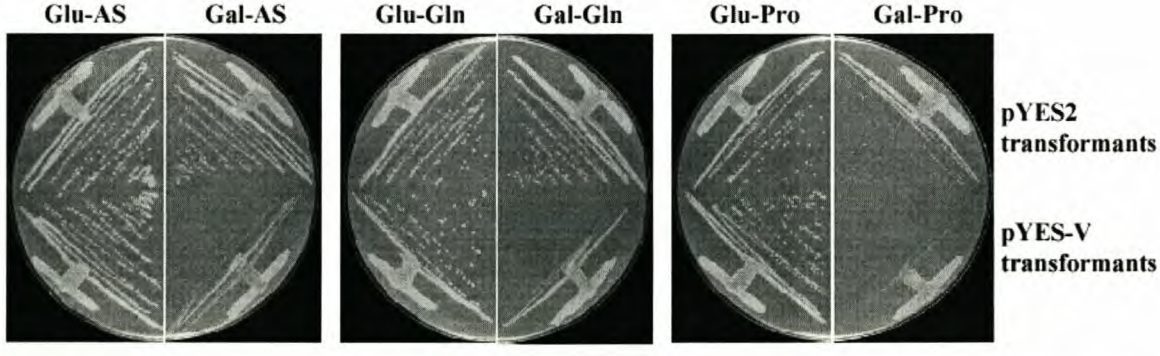

FIG. 6. **Overexpression of *VID30* negatively affects cell growth.** Growth of BY4742 transformed with pYES-V that overexpresses *VID30*; pYES2 was used as a negative control. Transformants were grown on YNB-2% glucose and YNB-2% galactose media containing either 0.1% ammonium sulphate (*Glu-AS* and *Gal-AS*), 0.1% glutamine (*Glu-Gln* and *Gal-Gln*) or 0.1% proline (*Glu-Pro* and *Gal-Pro*) as sole nitrogen sources. Ammonium sulfate- and glutamine-containing plates were incubated for 2 days and proline-containing plates for 4 days at 30 °C.

YNB-galactose-proline medium. Cells transformed with *GAL1-VID30* pYES-V grow like the control cells on YNB-glucose, irrespective of the nitrogen source supplied.

However, growth on all three nitrogen sources is inhibited when *VID30* is overexpressed (galactose as carbon source) (Fig. 6). These results indicate that controlled expression of *VID30* is essential for optimal cell growth.

Next, we examined the effect of *VID30* overexpression (Fig. 7, *top panels*) on the transcription of nitrogen-associated genes in cells provided with different nitrogen sources. If anything, Vid30p exhibits characteristics of a very modest negative regulator of NCR-sensitive gene expression in cells growing with proline as nitrogen source (Fig. 7*A*). There is somewhat more expression of *CAN1*, *DAL4*, *DAL5*, and *MEP2* observed in the wild type strain transformed with pYES2 than with pYES-V; *DAL4* expression is clearly most responsive. Two genes, *DAL1* and *DAL80*, did not respond to *VID30* overexpression (Fig. 7*A*). Overexpression of *VID30* did not detectably alter expression of any of these genes in cells grown under repressive conditions (with glutamine as the nitrogen source) (Fig. 7*A*). These results support the suggestions that: (i) Vid30p negatively regulates NCR-sensitive gene expression in cells grown with proline as nitrogen source, and (ii) Vid30p has no effect on the transcription of these genes in cells grown under repressive nitrogen conditions. These conclusions are consistent with those derived from the data in Fig. 3*A* where, by deletion, Vid30p was shown to be a negative regulator of *CAN1*, *DAL4*, *DAL5*, *MEP2*, *DAL1*, *GDH3*, and *DAL80* gene expression in proline-grown cells.

The expression profiles of the ammonia-glutamate-glutamine inter-conversion genes were more complex. In YNB-proline grown cells, *GDH1* expression slightly decreases, while the decrease in *GLT1* expression is dramatic; *GLN1* expression slightly increases (Fig. 7*B*). In contrast, *GDH2* expression increases rather markedly in proline grown cells overexpressing *VID30* (Fig. 7*B*). Expression of *GDH1* modestly increases, that of *GDH2* decreases slightly and that of *GLN1* and *GLT1* is not affected by *VID30* overexpression with glutamine as nitrogen source (Fig. 7*B*).

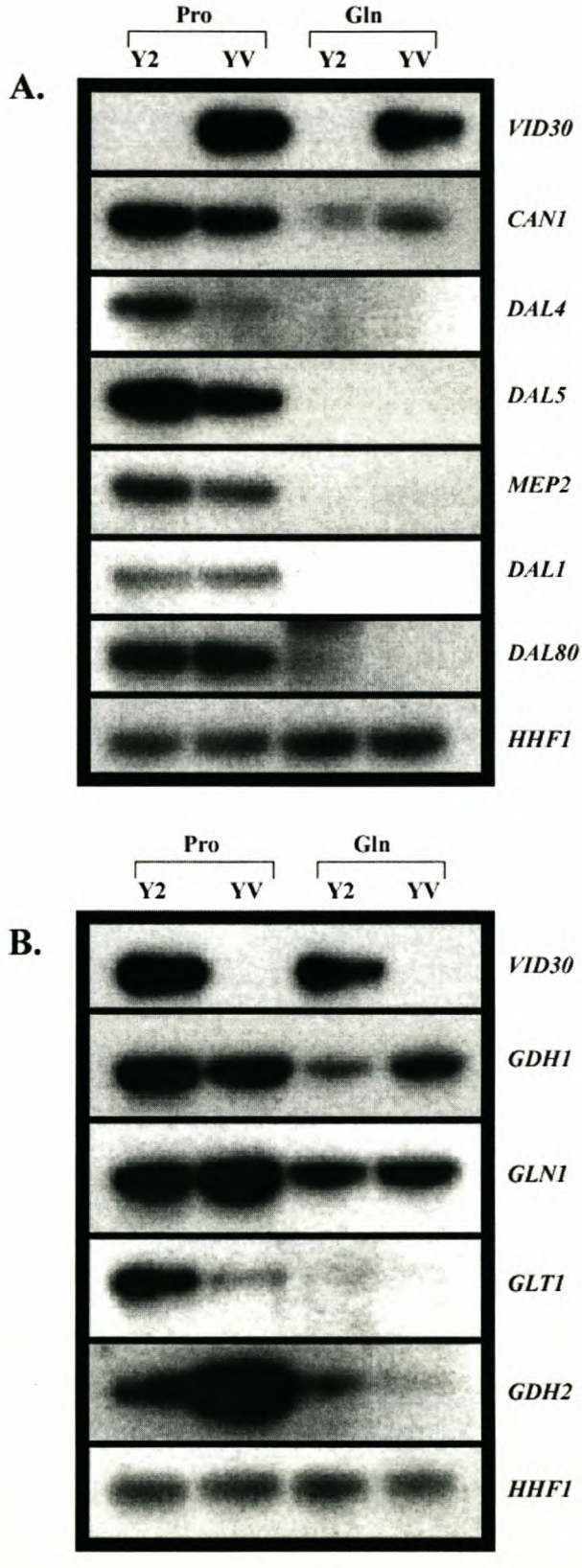

FIG. 7. **Overexpression of *VID30* affects the expression of genes associated with nitrogen metabolism.** Northern blot analysis of poly(A)$^+$ RNA (1 μg/lane) from transformants of BY4742 containing pYES2 (*Y2*) or pYES-V (*YV*) grown in YNB-0.1% proline (*Pro*) and YNB-0.1% glutamine (*Gln*) with 1.5% raffinose + 0.5% galactose as carbon source.

DISCUSSION

Data presented in this work demonstrate that Vid30p is a participant in the complex regulatory network controlling nitrogen metabolism in *S. cerevisiae*. *VID30* is among the genes whose expression is NCR-sensitive, regulated by Ure2p, and induced by rapamycin. The strength of regulation, however, is only modest to moderate when compared with that of *DAL5* or *DAL80* under similar circumstances. The more dramatic regulation of *VID30* expression occurs in response to ammonia. In low ammonia medium, *VID30* expression is remarkably high and rapidly decreases when the ammonia concentration in the medium is increased (Fig. 8). That *VID30* expression is enhanced in response to low ammonia rather than starvation is supported by the observation that it does not occur in similarly low concentrations of proline.

The effects of Vid30p on the expression of genes associated with nitrogen metabolism are quite complex. With proline as nitrogen source, Vid30p negatively regulates the nitrogen catabolic genes, *CAN1*, *DAL4*, *DAL5*, *MEP2*, *DAL1*, *DAL80*, and *GDH3*, but does not affect expression of *GDH1*, *GLN1*, or *GLT1*. There is a small positive effect on *GDH2*. With glutamine as the nitrogen source, the direction and strength of regulation is reversed. There is little if any effect of Vid30p on the regulation of *CAN1*, *DAL4*, *DAL5*, *MEP2*, *DAL1*, *DAL80*, and *GDH3*. In contrast, Vid30p is a significant negative regulator of *GDH1*, *GDH2*, *GLN1*, and *GLT1* expression. The most obvious difference between these two sets of genes is that the latter are associated with the interconversion of ammonia, glutamate, and glutamine.

Similar partitioning of these nitrogen metabolic genes is observed when the influence of Vid30p on their expression profiles was assayed in high and low ammonia. Since high ammonia is a strongly repressive condition, little if any expression was observed for *CAN1*, *DAL4*, *DAL5*, *MEP2*, *DAL1*, *DAL80*, and *GDH3*, and the presence or absence of Vid30p does not affect it. *GDH1*, *GLN1*, and *GLT1* were expressed in high ammonia, but again Vid30p did not affect their expression. Only for *GDH2* does Vid30p behave as a negative regulator. On low ammonia, Vid30p is a positive regulator of all genes except *GLN1* and *GDH2*, where it behaves as a negative regulator. Data obtained with cells overexpressing *VID30* are consistent with the patterns of regulation just summarized.

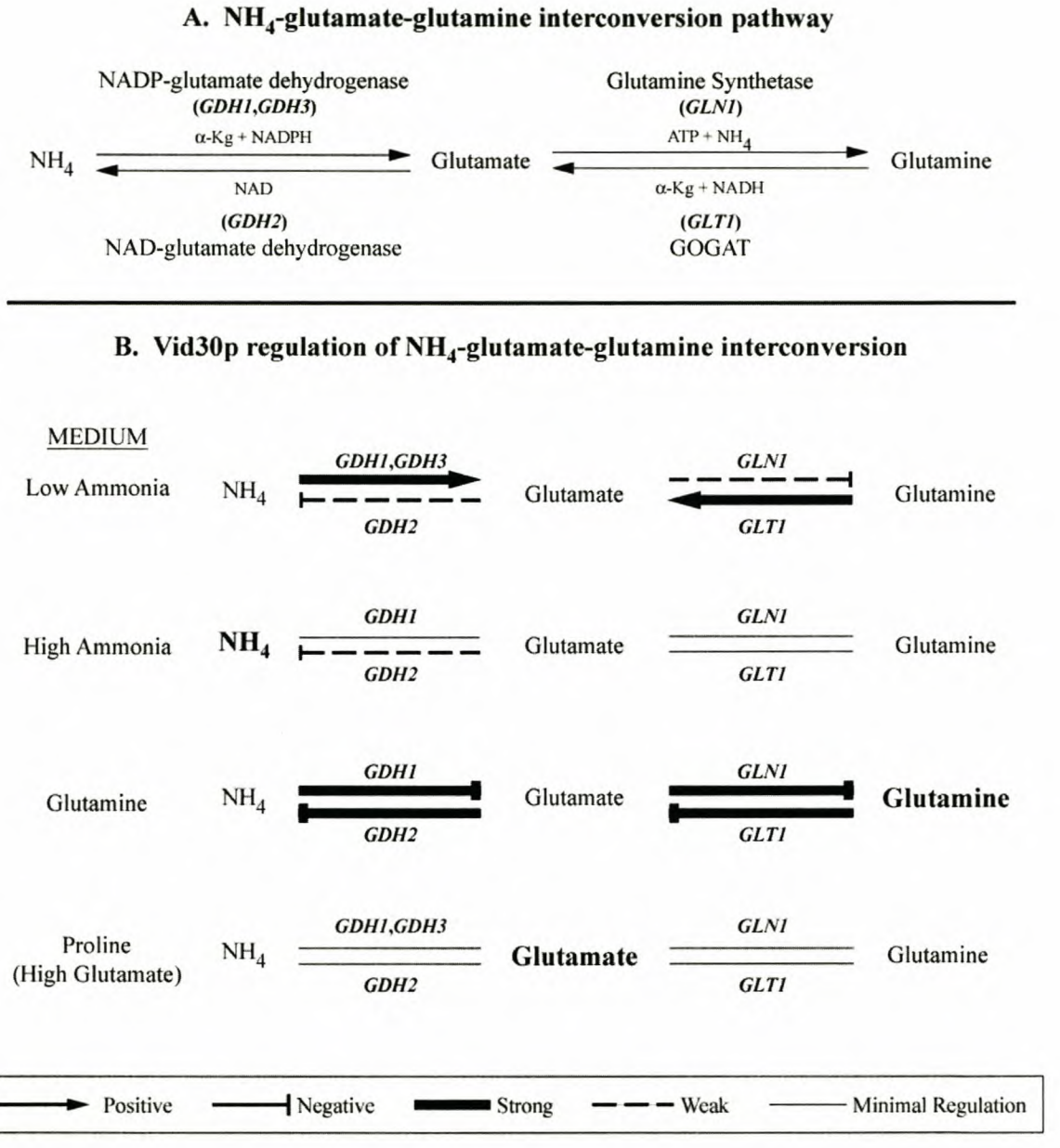

FIG. 8. **Working model of Vid30p regulation of nitrogen metabolism in *S. cerevisiae*.** *Arrows* and *bars* indicate that Vid30p function is positively or negatively regulating expression of the indicated genes. For example, Vid30p positively regulates *GDH1* and *GDH3* expression and negatively regulates *GDH2* expression when cells are cultured in low ammonia.

From these data, we conclude that Vid30p function most significantly influences the control of ammonia, glutamate, and glutamine interconversion (Fig. 8), functioning to keep metabolism directed towards glutamate formation especially when limiting ammonia is provided to the cells. The following reasoning supports this suggestion. The *GDH1*, *GDH2*, *GLN1*, and *GLT1* gene products can be functionally divided into two

groups: (i) enzymes that, respectively, catalyze conversion of ammonia and glutamine to glutamate, *GDH1*, *GDH3*, and *GLT1*, and (ii) those that, respectively, catalyze conversion of glutamate to ammonia and glutamine, *GDH2* and *GLN1*. *GDH1, GDH3*, and *GLT1* expression decreases markedly when a *vid30*Δ is grown in low ammonia, whereas *GDH2* and *GLN1* expression modestly increases. In other words, Vid30p modestly represses expression of genes whose products decrease the glutamate pool (*GDH2*, *GLN1*) and markedly activates expression of those whose products increase it (*GDH1*, *GDH3*, *GLT1*). These responses shift the interconversion reactions toward production of glutamate and away from ammonia and glutamine.

When cells are grown with high ammonia, *VID30* expression itself is strongly decreased, thereby decreasing its ability to serve as a regulator. Therefore, it is not too surprising that about the only thing Vid30p does in high ammonia is to repress expression of *GDH2*, whose product decreases the glutamate pool. The NCR-sensitive *GDH3* gene is not expressed under these conditions. In high ammonia, as in low, Vid30p function appears to be shifting the balance of metabolism towards glutamate. *GLT1*, *GLN1*, and *GDH1* expression is not demonstrably affected in high ammonia, while *GDH2* and *GLN1* do not greatly respond to a *vid30*Δ with low ammonia. This seeming contradiction, however, is more apparent than real. Recall that *GLT1* and *GLN1* can function in concert to form glutamate from ammonia. In this regard, it is pertinent to mention that the K_m for glutamine of the *S. cerevisiae* GOGAT (encoded by *GLT1*) is reported to be 0.29 mM (34). Such high concentrations of glutamine are much more easily achieved in high rather than low ammonia medium. By this reasoning, GOGAT functions along with the *GDH1* product to produce glutamate when environmental ammonia is high and when *GDH3*, whose product is responsible for one of the three routes to glutamate production, is largely not expressed due to its NCR sensitivity. If this suggestion is correct, GOGAT may be able to function quite differently in yeast than in bacteria where it, in collaboration with glutamine synthetase, is reported to be the primary route of ammonia assimilation (30). In summation, when ammonia is the nitrogen source, Vid30p function shifts nitrogen metabolism towards glutamate production in the face of many biosynthetic reactions that use glutamate to produce nitrogenous macromolecules.

The end products of nitrogen catabolic pathways associated with most poor nitrogen sources such as proline or allantoin are glutamate or ammonia, respectively, produced at a growth-limiting rate. Consistent with these physiological conditions, Vid30p function exhibits characteristics of a negative regulator for the expression of typical NCR-sensitive genes whose expression we measured when cells are provided with proline as the sole nitrogen source. It also is not particularly surprising that the ammonia-glutamate-glutamine interconversion genes are not highly regulated in proline medium, because the cell's glutamate requirement will be fulfilled more readily than either that for glutamine or ammonia. This derives from the fact that glutamate is the primary end product of proline catabolism. The situation is quite different when the nitrogen source is glutamine. In this case, the need for ammonia is much smaller and its supply is much greater. Furthermore, there is a good supply of glutamine and glutamate as well. Therefore, Vid30p function partially represses expression of all of the interconversion genes with *GLT1* being the least down-regulated when glutamine is provided as sole nitrogen source. Vid30p function does not appear to repress *GDH3* expression, but in this instance, Vid30p-mediated repression is not necessary, because *GDH3* expression is already highly repressed due to its NCR sensitivity. Although the data provide new insights into the nature of potential Vid30p functions, the mechanistic details of how these functions are accomplished remain to be elucidated, as do the identities of direct Vid30p targets.

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GENERAL DISCUSSION

We embarked on a study of the nitrogen-regulated gene expression in *Saccharomyces cerevisiae* by addressing the promoter-mediated regulation of nitrogen-sensitive genes and by studying the signal transduction required for the nitrogen-sensitive transcriptional response. The intergenic region separating the *DAL1* and *DAL4* genes was the target of the promoter study, while Vid30p was identified as a new regulator that is required to regulate the expression of various genes in low concentrations of ammonia.

DAL1 and *DAL4* were hypothesized and proved to be the first example of divergently transcribed genes in the nitrogen metabolism of *S. cerevisiae*. Detailed mutational analyses of the known nitrogen-related elements (Buckholz and Cooper, 1991; Yoo *et al.*, 1992) in the intergenic promoter region separating these genes revealed that all the predicted expression patterns could not be confirmed. Based on previous analyses of the other allantoin system genes, one would predict that elements most proximal to the core promoter of either of these genes would have the greatest impact on its transcriptional regulation. This is indeed the case for *DAL4* where *GATA4* and *GATA5* contribute the greatest to its expression. However, in the case of *DAL1*, *GATA1* does not contribute significantly to the transcriptional activation of this gene. To the contrary, this element seems to have a negative effect on *DAL1* expression. *GATAs2-4*, located in the central region of the intergenic region, are responsible for *DAL1* derepression. In addition, none of the 5 *GATAs* contribute equally to the expression of both genes. Thus, although *GATAs2-4* contribute significantly to the expression of both *DAL1* and *DAL4*, these contributions are not equal (van der Merwe *et al.*, 2001b).

Mutation analyses of the UIS_{ALL} elements also revealed contradictory patterns. *UIS8* is the UIS_{ALL} most distal and most proximal to the *DAL1* and *DAL4* core promoters, respectively. This element contributes the greatest to the induced expression of both genes. Again, the levels of induction supported by *UIS8* for both *DAL1* and *DAL4* are not equal (van der Merwe *et al.*, 2001b). The element sharing the greatest sequence similarity with the consensus UIS_{ALL} sequence, *UIS7*, was previously shown to bind Dal82p much stronger than *UIS8,* which has less similarity to the consensus sequence (Dorrington and Cooper, 1993). Thus, one would predict *UIS7* to be the major element

responsible for *DAL1* and *DAL4* induction. To the contrary, not only does this element not contribute significantly to the induction of either *DAL1* or *DAL4*, but it also has a negative effect on the expression of both genes (van der Merwe *et al.*, 2001b). Collectively these observations argue that predicted expression patterns of a specific gene based on the sequence homology of an element and the position of such an element in a given promoter should not be regarded as fact. More rigorous analyses should be done to draw accurate conclusions as to the function of a given element in the full context of its promoter.

The TOR signal transduction pathway senses environmental nitrogen and transduces the signal to the nucleus to elicit the suitable response for the organism to maximally benefit from its environment (Beck and Hall, 1999; Cardenas *et al.*, 1999; Hardwick *et al.*, 1999; Shamji *et al.*, 2000). Rapamycin specifically inhibits the function of the TOR proteins (Cardenas *et al.*, 1999; Cutler *et al.*, 1999; Kunz *et al.*, 1993). In a search for rapamycin-related molecules, we identified a relatively unknown protein, Vid30p[1], that functions to regulate the expression of various nitrogen-regulated genes. The transcription of the *VID30* gene itself is nitrogen-regulated and its transcription profile indicates that it functions in nitrogen-limiting conditions, when TOR is inactive, and specifically in the presence of low concentrations of ammonia. Transcriptional analyses of the *vid30*Δ mutant revealed that it functions by shifting the nitrogen metabolism towards the formation of glutamate in low ammonia concentrations (van der Merwe *et al.*, 2001a). A protein involved in nitrogen-regulated gene expression has therefore been successfully identified.

The mechanism by which Vid30p elicits its regulation is currently unclear. No possible target(s) of interaction and function have yet been identified. Also, the exact localization of this protein within the cell has not been determined. These questions will be targeted in future research.

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